COMPOSITION OF ENOLOGICAL NUTRIENTS AND THEIR EFFECT ON MALOLACTIC FERMENTATION

A Thesis

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ABSTRACT

Malolactic fermentation (MLF) is an important step in wine production, and can take place spontaneously or by induction, typically after the completion of alcoholic fermentation. MLF is known to reduce unwanted acidity and produce desirable aromas in some wine styles. Wine lactic acid bacteria (LAB) used for MLF have fastidious nutritional requirements, which may not be met by wine constituents, impeding MLF. Commercial nutrient preparations are available to assist malolactic fermentation, but little is known about their exact composition and ability to support bacterial growth and performance. The aim of this study was to carry out a detailed chemical analysis of enological and malolactic nutrients, and to evaluate their effect on the growth of commercial malolactic bacterial strains. Six commercial nutrients were analyzed for moisture content, turbidity, elemental composition, free amino nitrogen (FAN), free amino acids, total amino acids, vitamins, and fatty acids. Two *Oenococcus oeni* and one *Lactobacillus plantarum* strains were used, in combination with each of the six nutrients, for growth studies under anaerobic conditions in a model wine solution. The six nutrients varied significantly in individual analytes, and both bacterial growth and the conversion of malic acid by the MLF were determined by the type of nutrient used.

Keywords

Malolactic fermentation (MLF), wine lactic acid bacteria (LAB), enological nutrients, malolactic nutrients, yeast extract

BIOGRAPHICAL SKETCH

Alison Sudano is from a small town in Southern New Jersey, a suburb of Philadelphia, called Williamstown. She has always been interested in science and has had a passion for food since she was very young. Being raised in an Italian-American household, she was exposed to many cooking techniques and home winemaking at a young age. Some of her favorite hobbies are cooking and trying out new foods and trendy restaurants. Upon graduating from Williamstown High School, she originally wanted to pursue a career in physical therapy or sports medicine. Because of this, she decided to major in biology. Her career goals changed half-way through college when she learned about the field of food science. She obtained a Bachelor's of Science degree in biology from Rowan University, which is in Glassboro, NJ, close to her hometown. When it came time to decide on a university for her graduate studies, she thought Cornell University would be the perfect fit because of their Food Science – Enology program. Her graduate studies at Cornell started in August of 2011. Since then, she has enrolled in many courses such as Sensory Evaluation, Concepts of Product Development, and Winemaking Theory and Practice, to name a few, in addition to being an active member of the Cornell University Food Science Club, the Cornell University Viticulture and Enology Club, IFT, Western New York IFT, and the American Society for Enology and Viticulture (ASEV). She also was a graduate teaching assistant for the Winemaking Theory and Practice I and II Laboratories for the 2011-2012 academic year. Her research in the wine microbiology laboratory at Cornell focused on investigating aspects of wine lactic acid bacteria nutrition and performing compositional analysis on commercially available enological nutrients. Upon graduating with a Masters degree from Cornell, she hopes to work in the food science field in product development or food safety/quality assurance.

This work is dedicated to my parents, Frank and Maria Sudano, my sister, Madison Sudano, and my husband-to-be, James Viola for their unconditional love and support. They have always encouraged me to follow my dreams and never give up.

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CHAPTER 1: LITERATURE REVIEW

MALOLACTIC FERMENTATION AND ITS EFFECTS ON WINE

Malolactic fermentation (MLF) is a deacidification process in which dicarboxylic L-malic acid is converted into monocarboxylic L-lactic acid and CO_2 by the malolactic enzyme of lactic acid bacteria (LAB) (Figure 1.1) (Fugelsang, 1997; Lonvaud-Funel and Strasser De Saad, 1982; and Davis et al., 1985).

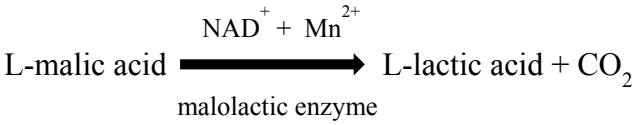

Figure 1.1: Malolactic fermentation mechanism.

MLF is a secondary fermentation that typically takes place concurrent to, or just after, alcoholic fermentation in wine, and is considered desirable for many red and some white wine styles, especially in regions where high acidity is a concern. Indigenous lactic acid bacteria, which can grow on wine grapes and contaminate winery surfaces, can be responsible for spontaneous malolactic fermentations (Fugelsang and Edwards, 2007). Traditionally, vinification practices have relied on the spontaneous MLF from naturally occurring LAB. However, due to the stressful environment wine presents, spontaneous MLF is rarely successful and is unpredicatable. To overcome these stressful conditions, starter cultures can be used to induce malolactic fermentations (Kunkee et al., 1964; Kunkee, 1991; Henschke, 1993; and Henick-Kling, 1993). MLF in wines is typically carried out by the bacterial strains *Oenococcus oeni* (formerly *Leuconostoc oenos*; Dicks et al., 1995), *Lactobacillus* spp. and *Pediococcus* spp. (Wibowo et al., 1985). Among these strains of LAB, there are homofermentative species and heterofermentative species. The homofermentative species produce lactic acid as the sole end product, while the heterofermentative species produce lactic acid, CO_2 and ethanol/acetate with

at least half of the end product of carbon being lactate (König and Fröhlich, 2009). Heterofermentative LAB utilize the pentose phosphate pathway, which is alternatively referred to as the phosphoketolase or phosphogluconate pathway (König and Fröhlich, 2009).

In addition to reducing the acidity of wines, MLF can contribute to aroma development in wines (Liu and Palone, 1998). Wine LAB are known to metabolize citric acid, which, along with malic and tartaric acids, is one of the main predominant organic acids in grape musts and wine (Lui, 2002). One of the most important effects of citrate fermentation is the production of diacetyl, an aroma compound with a buttery flavor note. In general, wines that have undergone MLF have higher concentrations of diacetyl (Martineau and Henick-Kling, 1995), which is considered to be a positive sensory characteristic if not present in excessive concentrations. A number of factors, such as bacterial strain, wine type, and sulphur dioxide and oxygen levels, can affect the final level of diacetyl that is produced in wine (Nielsen and Richelieu, 1999; Martineau and Henick-Kling, 1995). Wine LAB may also carry out other metabolic transformations, which can negatively influence wine quality, such as the production of biogenic amines or the carcinogenic ethyl-carbamate precursor citrulline (Lonvaud-Funel, 1999; Mira de Orduña et al., 2001). These by-products can also produce undesirable aromas. Also, it has been demonstrated that *O. oeni* might play a role in the production of histamine, which is known to be an allergen to humans (Lonvaud-Funel and Joyeux, 1994).

CHALLENGES OF MLF

LAB starter cultures are often selected based on their tolerance to stressful wine conditions. Wine pH, alcohol concentration, temperature, SO_2 concentration and organic acid content are some important factors known to affect bacterial growth (Wibowo et al., 1985; Henick-Kling, 1993; Davis, et al., 1988; Gockowiak and Henschke, 2003; and Versari, et al.,

1999). To encourage MLF, a wine pH of less than 3.5 is desireable. At lower pHs, it is more difficult for wine lactic acid bacteria to grow, but the growth of spoilage microorganisms is also inhibited. *O. oeni* is often chosen for the induction of MLF because of their tolerance of low pH levels (Henick-Kling, 1988; Versari et al., 1999).

High levels of sulfur dioxide (SO_2), which is often added during and at the end of alcoholic fermentation, can also inhibit the growth of wine lactic acid bacteria, but sensitivity can vary among species. The antimicrobial effect of SO_2 is greater as pH drops below 3.5, favoring the formation of free molecular SO_2, rather than the less-effective sulfite or bisulfite species (Gockawiak and Henschke, 2003). High alcohol content can also inhibit growth, especially with alcohol concentrations above 6%, with 14% (v/v) being the upper limit tolerated by most strains (Spano and Massa, 2006). Also, since wine lactic acid bacteria are mesophyllic and have an optimal growth temperature between 15° and 30°C, the temperature at which a fermentation takes place is limited (Versari et al., 1999, Spano and Massa, 2006).

The interaction between yeast and wine LAB can also have an effect on MLF, as some strains of *Saccharomyces cerevisiae* wine yeast are known to produce inhibitory metabolites. Yeast can produce SO_2, medium chain fatty acids and certain proteins which inhibit wine LAB (Capucho and San Romao, 1994; and Dick et al., 1992). Some yeast strains can produce less than 30 mg/l SO_2, although some have been reported to produce more than 100 mg/l (Rankine and Pocock, 1969; Eschenbruch, 1974; Dott et al., 1976; and Suzzi et al., 1985). This is a significant amount of SO_2 alone, especially since most winemakers treat wines with additional SO_2 when the alcoholic fermentation is complete. A previous study (Davis et al., 1988), performed using various strains of wine LAB, showed that growth was inhibited when levels of SO_2 present were higher than 64 mg/L.

NUTRITION OF WINE LAB

In addition to the many stress factors wine LAB encounter during fermentation, sluggish and stuck MLF are often caused by a lack of nutrient availability. Previous studies have shown that LAB have very stringent, and strain dependent, nutritional requirements for growth (Terrade and Mira de Orduña, 2009). These nutritional requirements can also have an effect on the efficiency with which lactic acid bacteria degrade malic acid in wine.

Free amino acids present in wine are used by lactic acid bacteria as a nitrogen source during malolactic fermentation. In a single omission study performed by Terrade, et al., lactic acid bacteria were found to require arginine and proline, as well as the branched amino acids isoleucine, leucine and valine (Terrade and Mira de Orduña, 2009). Proline is generally abundant in grape musts (Lehtonen, 1996; Spayd and Andersen-Bagge, 1996), and arginine is the most prevalent amino acid in certain grape varieties. Arginine is of special interest in wine production because it is degraded via the arginine deiminase pathway, leading to pH increases and ATP formation (Liu and Pilone, 1998; Mira de Orduña, 2001; and Tonon et al., 2001). An increase in pH can lead to wine spoilage and undesirable aromas arising from the production of biogenic amines and acetic acid (Lonvaud-Funel, 1991).

In wine, lactic acid bacteria also require certain vitamins for growth. Pantothenic acid and nicotinic acid are required by most species, and thiamine is required by all heterofermentative species. Other species may have individual requirements for folic acid, riboflavin, pyridoxal phosphate, p-aminobenzoic acid, biotin and B_{12} (König and Berkelmann-Löhnertz, 2009; Terrade and Mira de Orduña, 2009). Elements such as manganese and magnesium are also known to stimulate wine lactic acid bacteria growth (Terrade and Mira de Orduña, 2009). Figure 1.2 shows the results of previous work on wine LAB essential nutrients. Of the essential

nutrients displayed, amino acids and vitamins were found to be the most important for wine LAB growth (Terrade and Mira de Orduña 2009).

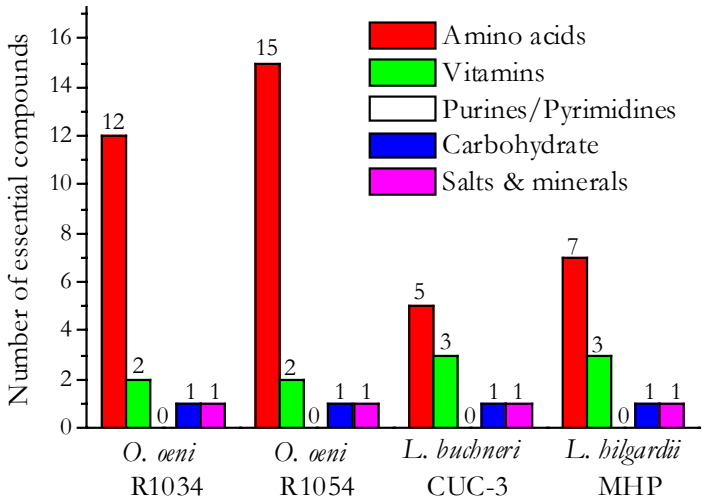

Figure 1.2: Number of essential nutrients for four wine LAB determined with a single omission technique.

Wine LAB also have the ability to metabolize carbohydrates present in wine. Some of these carbohydrates, such as sugars, may even be used as a substrate for growth. Depending on the strain of LAB and whether the strain is homofermentative or heterofermentative, sugars present in wine can be metabolized by different pathways. Sugars present in wine include a large range of both monosaccharides and disaccharides, with glucose and trehalose generally preferred over other sugars by wine LAB (Liu, 1990; Liu et al., 1995).

ENOLOGICAL NUTRIENTS

In nutritionally deficient musts or wines, nutritional requirements for both alcoholic fermentations (AF) and MLF can be met through the addition of exogenous nutrients. Both enological nutrients, for AF, and malolactic nutrients for MLF contain yeast and yeast-derived

ingredients, as well as ammonium salts and vitamins (FIVS-Abridge, 2012). There are over one hundred different commercial brands of nutrients currently available, claiming varying compositions and advantages. Only a few nutrients are recommended solely for malolactic fermentation, and are designed to ensure the fastidious nutrient requirements of malolactic bacteria are being met. While most commercial enological nutrients are designed specifically to support AF, they may be present in the wine during MLF, and their impact on LAB metabolism has not been fully investigated.

The actual composition of commercial enological and malolactic nutrients is largely unknown, and it is not certain which advantage either offer for malolactic fermentation. Most products consist of blends of inactivated yeast, yeast cell walls, vitamins, minerals, and metabolites produced through the yeast autolysis process, such as amino acids, peptides, proteins, polysaccharides, nucleotides and fatty acids (Zhang, 2003). However, nutrients do not carry content labels, making it difficult for users to know what constituents, and in what quantity, are included. In addition, the composition of yeast autolysates vary depending on yeast culture conditions (Guilloux-Benatier and Chassagne, 2003), making labeling difficult. However, the knowledge of nutrient composition is important, because legal limits exist for some constituents, and vary by country (Table 1.1) Regulations are relatively strict in the United States, Chile, and the European Union, while other countries, like Australia and New Zealand, take a broader best practices approach.

	USA	Australia	Canada	Chile	EU	New Zealand	OIV	South Africa
Ammonium Phosphate or DAP (g/l)	0.96	GMP	GMP	0.96	(DAP) 1 (expressed in salts), 7, or 0.3 for the second fermentation of sparkling wines, respectively	GMP	0.30	GMP
Calcium pantothenate (mg/L)	0.479	GMP	-	-	-	GMP	-	-
Potassium phosphate	-	GMP	GMP	-	-	-	-	-
Soy flour (g/L)	0.24	GMP	-	-	-	-	-	-
Thiamin(e) (mg/L)	0.60	GMP	-	0.60	0.60	-	0.60	GMP
Yeast, autolyzed (g/L)	GRAS	-	-	-	-	-	-	GMP
Yeast, cell wall/membranes of autolyzed yeast (g/L)	0.36	GMP	-	0.40	0.40	GMP	0.40	GMP

Table 1.1: Table of enological nutrient ingredients and their legal doses in some winemaking countries. Information is from the FIVS Abridge Database. (GMP = Good Manufacturing Practice, GRAS = Generally Regarded As Safe, blank space = no legal information available)

INACTIVE YEAST PREPARATIONS IN ENOLOGICAL NUTRIENTS

The main components of enological nutrients are inactive yeast preparations, which can be classified by production method into four types: inactive dry yeast, yeast autolysates, yeast hulls and yeast extracts. These inactive yeast products are obtained from yeasts by autolytic, plasmolytic or hydrolytic processes and then concentrated or dried to prepare a commercial formula (Münch et al., 1997; and Nagodawithana, 1992). Inactive dry yeast is obtained through thermal inactivation followed by drying. Yeast autolysates undergo an incubation step, allowing

enzymes to be released, in addition to thermal inactivation. Yeast hulls or walls are the insoluble component of yeast cell walls, with the cytoplasmic content of the cell removed. The use of inactive yeast preparations in the winemaking industry was derived from the food industry, where these products are widely used as flavoring and aromatizing agents to simulate meat, broth, or cheese-like flavors, and to aromatize snacks, soups and cheese products (Munch et al., 1997; and Nagodawithana, 1992).

Yeast extracts, the inactive yeast product most commonly used for enological nutrients, are produced through autolysis at 50°C in the presence of solvents or salts (Akins and Murphy, 1981; Chao et al., 1980; and Kelly, 1983). They consist of the soluble extract remaining after the total degradation of the cytoplasmic content, and are a mixture of peptides, amino acids, carbohydrates, and water soluble vitamins (Pozo-Bayón, 2009). Various production organisms, such as *Sacchromyces* spp. and *Candida* spp., can be used for yeast extracts, though extracts produced for wine supplementation are generally derived from *Saccharoymes cerevisiae* (Bridson and Becker, 1970).

Yeast extract is also commonly used as a component for cultivating microorganisms because of its low cost and rich content of various amino acids, peptides, water-soluble vitamins, growth factors, trace elements, and carbohydrates. However, the composition of yeast extract can vary due to the complex substrates used for production, poorly-controlled fermentation conditions used for yeast cultivation by manufacturers, and variations in downstream processes (Crueger and Crueger, 1989). Variability between different lot numbers of the same product can be great, and can lead to inconsistent performance, especially when enological nutrients are used for wine fermentation. One study claimed that different lots of yeast extract from the same

manufacturing process gave biomass and growth rate levels that varying by almost 50% (Potvin et al., 1997).

RESEARCH GOALS

In response to the need for better understanding of enological nutrient composition and their impact on malolactic fermentation, this research was designed to investigate the composition of selected enological and malolactic nutrients. More specifically, six commercial nutrients, four intended for MLF and two for AF, were analyzed for physical properties and chemical composition. These six nutrients were also added to support the growth of three strains of wine lactic acid bacteria used for malolactic fermentation. Malolactic fermentations were assessed using two strains of *Ooenococcus oeni* and one strain of *Lactobacillus plantartum* in a model wine solution. Both the compositional analyses and growth studies pairing wine lactic acid bacteria cultures with commercial enological nutrients can provide data to assist winemakers in the implementation and quality control of malolactic fermentation.

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CHAPTER 2: COMPOSITION OF ENOLOGICAL NUTRIENTS AND THEIR EFFECT ON MALOLACTIC FERMENTATION

ABSTRACT

Wine lactic acid bacteria have fastidious nutritional requirements, which may not be met by wine constituents, impeding malolactic fermentation (MLF). Commercial nutrient preparations are available to assist malolactic fermentation, but little data is available regarding their exact composition and ability to support bacterial growth and performance. The aim of this study was to carry out a detailed chemical analysis of enological and maoloactic nutrients, and to evaluate their effect on the growth of commercial malolactic bacterial strains. Six commercial nutrients were analyzed for moisture content, turbidity, elemental composition, free amino nitrogen (FAN), free amino acids, total amino acids, vitamins, and fatty acids. Two *Oenococcus oeni* and one *Lactobacillus plantarum* strain were used as organisms for growth studies, in combination with each of the six nutrients, under anaerobic conditions in a model wine solution. The six nutrients varied significantly in concentration of some individual analytes, such as phosphorous, potassium, and leucine, and bacterial growth, along with the conversion of malic acid by the MLF, were determined by the type of nutrient used. An ANOVA test performed for the growth study using a modified model wine suggested that growth level is dictated by bacterial strain, enological nutrient, stage of fermentation, and combinations of these three variables.

Keywords

Malolactic fermentation (MLF), wine lactic acid bacteria (LAB), enological nutrients, malolactic nutrients, yeast extract

Introduction

Malolactic fermentation is a secondary fermentation that typically takes place after alcoholic fermentation in wine. During malolactic fermentation, malic acid is converted to lactic acid through decarboxylation (Fugelsang and Edwards, 2007). This results in less acidic, more palatable wines. Lactic acid bacteria may also contribute to aroma development in wines (Liu and Palone, 1998). Indigenous lactic acid bacteria, which can grow on wine grapes and contaminate winery surfaces, can be responsible for spontaneous malolactic fermentations (Fugelsang and Edwards, 2007), though many producers use starter cultures to induce malolactic fermentations. Previous studies have shown that lactic acid bacteria have very stringent nutritional requirements for growth, which can be strain dependent (Terrade and Mira de Orduña, 2009). These nutritional requirements can also have an effect on the efficiency with which lactic acid bacteria degrade malic acid in wine.

An important nutritional requirement for wine lactic acid bacteria is available nitrogen. Free amino acids present in wine are used by lactic acid bacteria as a nitrogen source during malolactic fermentation. In a single omission study performed by Terrade, et al., lactic acid bacteria were found to require arginine and proline, as well as the branched amino acids isoleucine, leucine and valine (2009). Proline is generally abundant in grape musts (Lehtonen, 1996; Spayd and Andersen-Bagge, 1996), and arginine is the most prevalent amino acid in certain grape varieties. Arginine is especially important because it is degraded via the arginine deaminase pathway, leading to pH increases and ATP formation (Liu and Pilone, 1998; Mira de Orduña, 2001; and Tonon et al., 2001). An increase in pH can lead to wine spoilage and undesirable aromas which arise from the production of biogenic amines and acetic acid (Lonvaud-Funel, 1991).

In wine, lactic acid bacteria also require certain vitamins for growth. Pantothenic acid and nicotinic acid are required by most species, and thiamine by all heterofermentative species. Other species may have individual requirements for folic acid, riboflavin, pyridoxal phosphate, p-aminobenzoic acid, biotin and B12 (König and Berkelmann-Löhnertz, 2009; Terrade and Mira de Orduña, 2009). Elements such as manganese and magnesium are also known to stimulate lactic acid bacteria growth in wine (Terrade and Mira de Orduña, 2009).

Given their high nutritional needs, lactic acid bacteria are often limited by deficiencies in essential nutrients. Some factors, like high levels of C_{10} and C_{12} fatty acids, have been proven to hinder growth of wine lactic acid bacteria species *O. Oeni*, while other fatty acids, such as C_{18}, $C_{18:1}$, and $C_{18:2}$, can increase cell biomass during a fermentation (Guilloux-Benatier et al., 1998). In nutrient deficient musts or wines, nutritional requirements can be met through the addition of exogenous nutrients before or during malolactic fermentation. Commercial malolactic nutrients may contain inactivated dried yeast and yeast-derived ingredients, as well as ammonium salts and vitamins (FIVS-Abridge, 2012). While there are over one hundred different commercial brands of enological nutrients currently available, designed to support yeast during alcoholic fermentation, only a small number are specifically offered for malolactic fermentation.

The actual composition of commercial enological and malolactic nutrients is largely unknown, and it is not certain what advantages they offer during malolactic fermentation. Most products consist of blends of inactivated yeast, yeast cell walls, vitamins, and minerals, as well as metabolites produced through the yeast autolysis process, such as amino acids, peptides, proteins, polysaccharides, nucleotides and fatty acids. However, nutrients do not carry content labels, making it difficult for consumers to know what constituents, and in what quantity, are included. In addition, the composition of yeast autolysates vary depending on yeast culture

conditions (Guilloux-Benatier and Chassagne, 2003), making labeling difficult. However, knowledge of nutrient composition is important because legal limits exist for some constituents, and vary by country.

In response to the need for better understanding of nutrient impact on malolactic fermentation, this research was designed to determine the composition of selected enological and malolactic nutrients. More specifically, six commercial nutrients, four designed to support malolactic fermentation, were analyzed for physical properties and chemical composition. These six nutrients were tested in malolactic fermentation trials to determine their impact on the growth of three strains of commercial wine lactic acid bacteria. Malolactic fermentations were performed using two strains of *Oenococcus oeni* and one strain of *Lactobacillus plantartum* in a "model wine" solution. Both the compositional analyses and growth studies pairing wine lactic acid bacteria cultures with commercial nutrients can provide data to assist winemakers in the implementation and quality control of malolactic fermentation.

MATERIALS AND METHODS

Enological Nutrients

Six commercial nutrients were donated by Lallemand (Monteral, QC.), four recommended for malolactic fermentation and two for alcoholic fermentation. The nutrients are labeled as Nutrients 1-6; Nutrients 3 and 4 are sold commercially for alcoholic fermentations, are intended to be added at the end of alcoholic fermentation. Nutrients 1, 2, 5 and 6 are designed specifically to support MLF.

Model Wine Solutions

For analysis, nutrients were dissolved in model wine, a hydroalcholic solution consisting of various acids and sugars at levels mimicking those found in wine (Table 1). For the free amino acid analysis and one of the growth studies, a modified model wine solution with higher pH, lower ethanol content, and higher levels of glucose and fructose was used to encourage growth of the three lactic acid bacteria strains. The alcohol content of both model wine solutions were determined using an Alcolyzer (Anton Paar GmbH, Austria). All reagents were analytical grade and obtained from Thermo Fisher Scientific (Pittsburgh, PA) or Sigma, Aldrich Co. (St. Louis, MO).

	Hydroalcoholic Solution for UHPLC Amino Acid Analysis and Growth Study (modified model wine)	Hydroalcoholic Solution for Other Compositional Analyses and Preliminary Growth Study
pH	3.5	3.2
L-Malic acid (g/L)	3.5	3.5
Citric acid (g/L)	0.5	0.5
Tartaric acid (g/L)	3.0	3.0
Fructose (g/L)	5.0	1.0
Glucose (g/L)	5.0	0.5
Ethanol (v/v)	8.0%	12.5%

Table 2.1: Hydroalcoholic solutions ("model wine") used for dissolving nutrients during each compositional analysis.

Compositional Analyses

All nutrients were analyzed for moisture content, turbidity, elemental composition, free amino nitrogen (FAN), free amino acids, total amino acids, vitamins, and fatty acids.

Moisture Content

Moisture content was determined using a modular moisture analyzer with an infrared quartz cylinder heating system with controlled airflow and a monolithic electronic precision balance with thermal isolation (Mark 3, Sartorius Omnimark, Tempe, AZ). The balance was calibrated

using both the internal calibration function as well as ASTM Class 150 g and 100 g external standards. A 2 g sample of each nutrient was weighed out on the moisture analyzer plate, and the analysis was performed in duplicate.

Turbidity

In order to evaluate the particulate quality of the nutrients and estimate solubility, 200 mg/L of each nutrient was dissolved in the model wine solution. The turbidity was measured with a Hach turbidimeter (Loveland, CO) calibrated with 0.1 – 100 NTU turbidity standards. Following this intial turbidity measure, solutions were centrifuged for 5 min at 3,800 rpm (Centra CL2 Thermo IEC Centrifuge, Madoon Heights, MA), and their turbidity measured again to distinguish turbidity caused by colloidal matter from that including larger, sedimentable particles.

Elemental Analysis

Elemental analysis was carried out by the Soil and Nutrition Laboratory at Cornell University. Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was used after microwave (Microwave Mars-Xpress, CEM Corporation, Matthews, NC) assisted acid (HNO_3) digestion of nutrient samples provided (EPA Method 3015-6010).

Glutathione Content

Analysis of total glutathione was carried out using a combined enzymatic/colorimetric test with glutathione reductase and DTNB (Cayman Chemical, Ann Arbor, MI). For this, 100 mg of nutrients were dissolved in 500 mL model wine, with stirring for 15 min, to reach a concentration of 200 mg/L. Once nutrients had dissolved, 50 mL of each solution was centrifuged at 3800 rpm for 10 min. The supernatant was sterile filtered (0.22 μm, nylon, Millipore, Billerica, MA) and analyzed directly, or in 1:2 and 1:10 dilutions. Except for Nutrient 6, where the 1:10 dilution was used, all values reported originate from undiluted samples.

Glutathione analysis was performed a second time for all nutrients after the glutathione content in Nutrient 6 was reduced by the sponsor.

Free Amino Nitrogen (FAN)

Analysis of free amino nitrogen was carried out using a modified NOPA (o-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric) method based on Dukes and Butzke (1998), using isoleucine as a standard for nitrogen concentration. Nutrient solutions were prepared at a concentration of 2.0 g/L by dissolving 500 mg of each nutrient in 250 mL hydroalcoholic solution for 24 hr (one solution for each nutrient), then sterile filtering through a 0.2 μm filter. This concentration was 10 times the recommended concentration of 200 mg/L, and was chosen after a preliminary FAN analysis at the lower concentraion returned results that were below the limit of detection. In variance from the Dukes method, 25 μL of standard or filtered sample was added to 1.40 mL of NAC buffer in a 10 mm cuvette, and the absorbance was measured at 335 nm (A_1). Upon measuring the absorbance a first time, 10 μL of OPA reagent was added to the cuvette. The next absorbance measurement was taken 5 minutes after the addition of the OPA reagent (A_2). A_2 was subtracted from A_1 to determine the final absorbance of the sample and the overall concentration of free amino nitrogen. Since the concentration of each sample was 10 times the recommended concentration of 200 mg/L, results were calculated by dividing the determined concentration of nitrogen by 10.

Ammonium Content

Ammonia analysis was performed with an ion selective electrode (ISE) (Cole Palmer, Vernon Hills, IL) on a 200 mg/L nutrient solution made by dissolving 100 mg of nutrient in 500 mL of model wine using the Dukes and Butzke YAN method (1998). Ammonium standards, ranging from 0-200 mg/L ammonium, were made using ammonium chloride.

Free Amino Acid Analysis by UHPLC

An ultra high pressure liquid chromatography system (Shimadzu, Canby, OR) consisting of a binary LC-20AD XR pumping unit, a DGU-20A3 degasser, a SIL-20AC XR autosampler, a CTO-20AC column oven, and a RF 10A XL flourescence detector, were used for separation and analysis of derivatized amino acids in the enological nutrients. Data acquisition and analysis was performed with the LCSolution software (1.23) (Shimadzu, Canby, OR). The method used for this free amino acid analysis was adapted from Shimadzu Method No. L432. This analysis consisted of a binary gradient (Table 2.2), where solvent A was a 20 mmol/L potassium phosphate buffer adjusted to pH 6.9 using sodium hydroxide, and solvent B a 45/40/15 solution of HPLC grade acetonitrile/HPLC grade methanol/ASTM Class 1 water (Arium 611UV, Edgewood, NY) . All solvents were filtered prior to use (0.22 µm, nylon, Millipore, Billerica, MA). Separation occurred on a YMC Triart C18, 1.9 µm reverse-phase column (75 mmL. x 3.0 mmL.D., 1.9 µm, YMC Co., Ltd.) with a EXP Triart C18 guard cartridge (5 mmL. x 3.0 mmL.D., 12 µm, YMC Co., Ltd).

HPLC Reagents

Derivatization reagents o-Phthaldialdehyde (OPA), 3-Mercaptopropionic Acid (3-MPA), and Fluorenylmethyloxycarbonyl Chloride (FMOC-Cl) were used for amino acid fluorescence in both the standards and samples. The OPA derivatization reagent was made by dissolving 20 mg of OPA in 10 mL of a 0.1 mol/L borate buffer (pH 9.2). The 3-MPA derivatization reagent was made by adding 10 µL of the 3-MPA to 10 mL of the same borate buffer (pH 9.2). The FMOC-Cl derivatization reagent was made by dissolving 20 mg of FMOC-Cl in 100 mL of HPLC grade Acetonitrile (Fisher Scientific, Fair Lawn, NJ). For separation of most of the amino acids, a commercially prepared combined standard (Amino Acid Standard H, Thermo Scientific,

Rockford, IL), which was prepared in 0.1 N HCl, was used. An additional combined standard was used for the analysis of Glutamine, Asparagine, Tryptophan, Citrulline, and Ornithine, the amino acids not present in the commercially prepared combined standard. Standards ranged from 2.5 to 30 µmol/L. Norvaline was used as an internal standard at a concentration of 25 µmol/L for each standard and sample.

Sample analysis

A solution of each nutrient dissolved at either 300 mg/L or 500 mg/L in the modified model wine was used for UHPLC analysis. Although the recommended dosage for these nutrients is 200 mg/L, a higher concentration of the nutrient was used for sample preparation to ensure visibility of amino acid peaks. Once dissolved, the samples were sterile filtered (0.22 µm, nylon, Millipore, Billerica, MA) before analysis. This analysis was performed three times, once with the commercially prepared combined standard with the nutrient samples at a concentration of 300 mg/L, once with the standard consisting of the five additional amino acids with the nutrient samples at a concentration of 300 mg/L, and again with the commercially prepared combined standard with the nutrient samples at a concentration of 500 mg/L, and with a higher addition of the FMOC-Cl derivatization reagent for the purpose of analyzing for proline. Proline was the only amino acid that was derivatized solely by FMOC-Cl and elutes at a different wavelength than other amino acids.

Derivatization

All standards and samples were derivatized by hand. First, 225 µL of the 3-MPA solution was added to a sample vial, followed by 110 µL of the OPA solution, and 37. 5 µL of the standard or sample. This mixture was allowed to incubate at room temperature for 3 minutes. After the three

minutes, either 10 µL or 50 µL of the FMOC-Cl solution was added, and the mixure was allowed to rest at ambient temperature for another 5 minutes before loading the sample vial onto the autosampler.

Mobile Phase	A: 20 mmole/L Potassium Phosphate Buffer (pH 6.9) B: 45/40/15 Acetonitrile/Methanol/Water
Program	B Concentration 11% →13 % (0-3.00 min) →31% (5.00 min) →37% (7.50 min) →70% (10.00 min) →100 (14.00-15.00 min) →11% (20.00 min)
Flow Rate	0.8 mL/min
Column Temperature	35°C
Injection Volume	1 µL
Detection	Excitation at 350 nm Emission at 450 nm → Excitation at 266 nm Emission at 305 nm (9.85 min)

Table 2.2: Analytical conditions for Free Amino Acid analysis by UHPLC.

Free Amino Acid Analysis by Ion-Exchange Chromotography

A second free amino acid analysis was performed by ion-exchange chromotography on a Hitachi 8900 amino acid analyzer (Hitachi High-Technologies America Inc., Schaumburg, IL) using an ion-exchange column (Transgenomic, Omaha, NE) at the University of California Davis Proteomics Core Center (Davis, CA). Samples of the six nutrients dissolved in the modified model wine solution at a concentration of 2 g/L, and post-fermentation supernantant samples of all fermentations, were analyzed in duplicate. All samples were sterile filtered (0.22 µm, nylon, Millipore, Billerica, MA), and 200 µL of each sample was precipitated with 50 µL of 10% sulfosalicylic acid to remove any intact proteins. A vortex was used to mix the samples, which were allowed to sit at ambient temperature for 15 minutes before 100 µL was transferred to a microcentrifuge tube and diluted with lithium-diluent spiked with AE-Cys (used as an internal standard) to reach a final sample dilution of 1:2.5. Samples were then centrifuged (Eppendorf,

Hauppauge, NY) at 16 rcf for 5 minutes, and 50 µL was injected on the column. Samples were analyzed at 570 nm (Vis1) and 440 nm (Vis2), and a commercial amino acid standard (Sigma, St. Louis, MO) was used as a control. The amino acid analyzer used lithium-based buffers and a post-column reaction with ninhydrin, both from Hitachi. Tryptophan was not quantified using this method.

Total Amino Acid Analysis by Ion-Exchange Chromatography

A total amino acid analysis was also performed by ion-exchange chromotography on a Hitachi 8800 amino acid analyzer (Hitachi High-Technologies America Inc., Schaumburg, IL) at the University of California Davis Proteomics Core Center (Davis, CA). The system utilized constant boiling HCl (hydrolysis) (ThermoScientofic, Rockford, IL) and an ion-exchange column (Transgenomic, Omaha, NE). Samples of the six enological nutrients dissolved in the modified model wine solution at a concentration of 2 g/L and post-fermentation supernantant samples of all fermentations were analyzed in duplicate. All samples were sterile filtered (0.22 µm, nylon, Millipore, Billerica, MA), and 200 µL of sample was dried. After drying, a liquid phase hydrolysis step was performed using 6N HCl and 1% phenol at 110ºC for 24hr. After this 24 hr hydrolysis step, the sample was allowed to dry again. The sample was then diluted with sodium-diluent (Pickering Laboratories, Mountain View, CA) spiked with NorLeucine (used as an internal standard) to reach a final sample dilution of 1:3. Samples were then centrifuged (Eppendorf, Hauppauge, NY) at 16 rcf for 5 minutes, and 50 µL was injected on the column. A commercial amino acid standard (Sigma, St. Louis, MO) was used as a control.

Vitamin and Fatty Acid Analysis

Eleven fatty acids and fourteen vitamins and vitamin dervatives were analyzed at Metabolic Discoveries GmbH in Potsdam, Germany using ultra high-pressure liquid

chromatography (UHPLC-ESI-QTOF 6540, Agilent, Santa Clara, CA). Fat-soluble vitamins and fatty acids were sepearated on a C18 column with sub 2 μm particles, and water-soluble vitamins on an HILIC stationary phase column. Preliminary methods testing was performed using the company's standard protol for Gas Chromatography (HP Agilient 7890A) /Mass Spectrometry (Agilent 5975C Series, Agilient, Santa Clara, CA) (GS/MS) and Liquid Chromotography/Mass Spectrometry (Agilent, Santa Clara, CA) (LC/MS) (Metabolic Discoveries GmbH, Potsdam, Germany) with MassHunter Software to optimize sample concentration and assess potential performance differences, though no significant differences were found (data not shown.) The mobile phase used for this pre-test was made of water, an organic solvent and a modifier to enhance ionization.

Prior to analysis, 20 g/L enological nutrients were extracted for 24 hours in the model wine solution at ambient temperature and in a dark environment to ensure the stability of the analytes. This concentration is 100 times higher than the recommended addition of the enological nutrients, and was chosen because results from preliminary analysis showed that 20 g/L was optimal for peak visibility. Two calibration curves were prepared for water-soluble and fat-soluble compounds. Samples were injected in duplicate, and run in positive and negative ionization mode on a column for polar compounds and a reversed phase system.

Lactic Acid Bacteria Growth Studies

Microorganisms

Commercial lactic acid bacteria strains *Oenococcus oeni* R1105, *Oenococcus oeni* R1124, and *Lactobacillus plantarum* R1122 were donated by Lallemand Inc. (Monteral, QC). Bacteria were stored in a 50% glycerol solution at -80ºC until needed.

Growth Media

All bacteria were pre-grown in AMRS medium consisting of 200 mL sterile filtered (0.22 μm, nylon, Millipore, Billerica, MA) apple juice with added ascorbic acid (Wegman's, Rochester, NY) and 55 g Difco Lactobacilli MRS broth (Becton, Dickinson, and Company, Sparks, MD), adjusted to pH 5.5 with KOH or HP_3O_4 and brought to 1L with distilled water. Fermentations were performed in model wine solutions, each containing one of the six nutrients.

Washing Buffer

Before inoculation, the bacteria were centrifuged at 3,800 rpm for 5 minutes (Centra CL2 Thermo IEC Centrifuge, Madoon Heights, MA) in the AMRS broth and washed with a NaHT (sodium hydrogentartrate) washing buffer. A Tween 80 (5% w/v) solution was made by dissolving 5 g Tween 80 in 100 mL of distilled water. A mineral solution was made by dissolving 20 g $MgSO_4 \cdot 7\ H_2O$ and 5 g $MnSO_4 \cdot 4\ H_2O$ in 100 mL of distilled water. The 50 mM NaHT washing buffer was then made by dissolving 7.5 g of tartaric acid, 1 mL of the Tween 80 solution, and 5 mL of the mineral solution, adjusting the pH to 4.0 with NaOH, bringing the volume to 1 L with distilled water.

Preliminary Work

All three bacterial strains were pre-grown in 25 mL AMRS medium in 50 mL test tubes at 30°C. Upon reaching stationary phase, cells were washed and resuspended twice in the NaHT washing buffer. They were then added at two concentrations to the model wine solution with a pH of 3.2 and ethanol content of 12.5% (v/v) (Table 2) in order to achieve 1 x 10^5 CFU/ml and 1 x 10^6 CFU/ml. Each of the six nutrients was then added at a concentration of 200 mg/L. Pimaricin, used as a fungicide, was added at a final concentration of 50 mg/L. Duplicate, 8 mL fermentations were performed for each nutrient X strain pairing in 15mL test tubes. Bacterial

growth was monitored through optical density measurements taken every 48 hrs using a spectrophotometer set at 600 nm (Pharmacia LKB Novaspec II UV/VIS, Piscataway, NJ). Fermentations were repeated in a scaled-up volume of 100 mL using the same inoculation rates and concentrations of nutrient and pimaricin. For both studies, fermentation vesssels were kept in a temperature-controlled anaerobic chamber (Coy Laboratory Products, Grass Lake Charter, MI) at 20°C.

Growth Study with Modified Model Wine

Fermentation Set-up

After the "scaled up" experiment displayed little to no growth, another growth study was done using a modified model wine with a pH of 3.5 and an ethanol content of 8.0% (v/v) (Table 2). Again, all three strains were pre-grown in 25 mL AMRS medium in 50 mL test tubes at 30°C. Upon reaching stationary phase, bacteria were washed and resuspended twice in the NaHT washing buffer. They were then added at 1 x 10^6 CFU/ml to 10 mL fermentations performed in 15 mL test tubes. The nutrient concentration was increased from 200 mg/L to 2.0 g/L and the pimaricin concentration increased from 50 mg/L to 150 mg/L. A control, consiting of bacteria without the nutrient addition, was also used. As in previous studies, fermentations were performed in a temperature-controlled anaerobic chamber (Coy Laboratory Products, Grass Lake Charter, MI) at 20°C. Viable cell plating on AMRS plates was used to determine growth of the bacteria at the time of inoculation, two days after inoculation, and at the completion of fermentation. The optimal density was not recorded because the high concentration of nutrient added to the fermentation, and subsequent turbidity, made it difficult to achieve an accuarate growth reading.

L-Malic Acid Assay

The degradation of malic acid in the fermentation samples was analyzed every three days using a malate dehydrogenase enzymatic assay; malolactic fermentation was considered complete when there was no l-malic acid present.

For this assay, a buffer was made by by dissolving 3.78 g glycine and 5.17 g hydrazine sulfate in water and the pH was adjusted to 9.0 with sodium hydroxide pellets. Once the pH was adjusted, 0.2 g of NAD was added and the volume was brought up to 100 mL. L-malic acid was used as the standard. To perform the assay, 80 µL of standard or sample was added to a cuvette along with 1.0 mL of the buffer. The absorbance was recorded (A_1) with a spectrophotometer and SpectraSuite spectrophotometer software (Ocean Optics, Dunedin, FL) at 340 nm, 10 µL of the MDH enzyme (6,000 U/mL) was added to the cuvette, and the solution was allowed to incubate for 1 hour at room temperature. The absorbance was recorded again (A_2) after the incubation period. A_1 was substracted by A_2 to determine the change in absorbance. A calibration curve using 7 standards from 5-350 mg/L was created using L-Malic acid, and used to calculate the L-malic acid concentratrion of the unknown samples based on the change in absorbance. Samples were diluted 1:10 before analysis to keep values in the range of the standard curve.

Statistics

Analysis of variance (ANOVA) was performed using JMP statistical software (JMP Pro 10, SAS Institute Inc., Cary, NC) to determine differences among nutrient composition and among viable cell counts in fermentation trials. More specifically, a fixed effects test was performed to display results of overall variable effects on growth level.

RESULTS

Compositional Analyses

The following results represent components present in a 200 mg/L enological or malolactic nutrient addition, the addition recommended for wine production.

Moisture Content

Nutrient moisture content ranged from 4.975 – 7.325%, with Nutrient 6 showing the highest content (Table 3).

Nutrient	Moisture Percentage	+/- Standard Error
1	4.975	0.0985
2	5.805	0.196
3	5.346	0.303
4	5.6625	0.0135
5	5.281	0.035
6	7.3255	0.2385

Table 2.3: Moisture content analysis results for six enolgical or malolactic nutrients.

Turbidity

Nutrient 5 had a higher concentration of turbidity-causing non-solubles, while Nutrient 6 was extremely soluble. Centrifugation and separation of the sediment led to more homogeneous supernatant.

Nutrient	Before Centrifugation (NTU)	After Centrifugation (NTU)
1	24.6	2.7
2	21.8	3.9
3	25.5	9.8
4	29.2	2.5
5	52.9	3.4
6	5.5	4.7

Table 2.4: Turbidity analysis results for six enological and malolactic nutrients both before and after centrifugation.

Elements

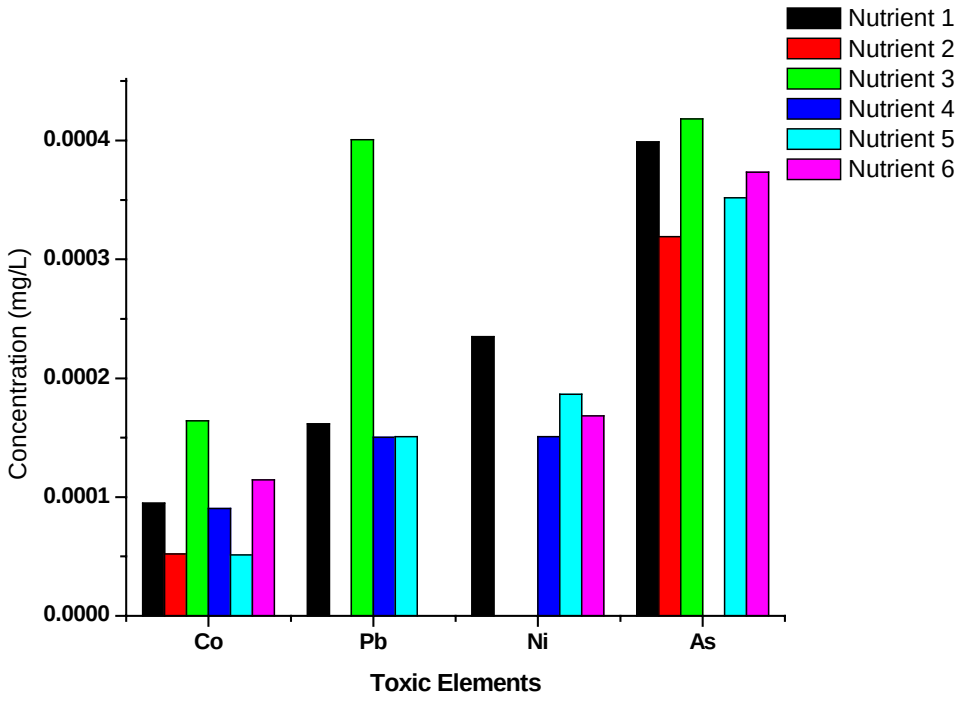

Figure 2.1: Elemental profile of cobalt, lead, nickel, and arsenic in commercial enological and malolactic nutrients. Mean concentrations are displayed for the recommended nutrient addition, which is 200 mg/L.

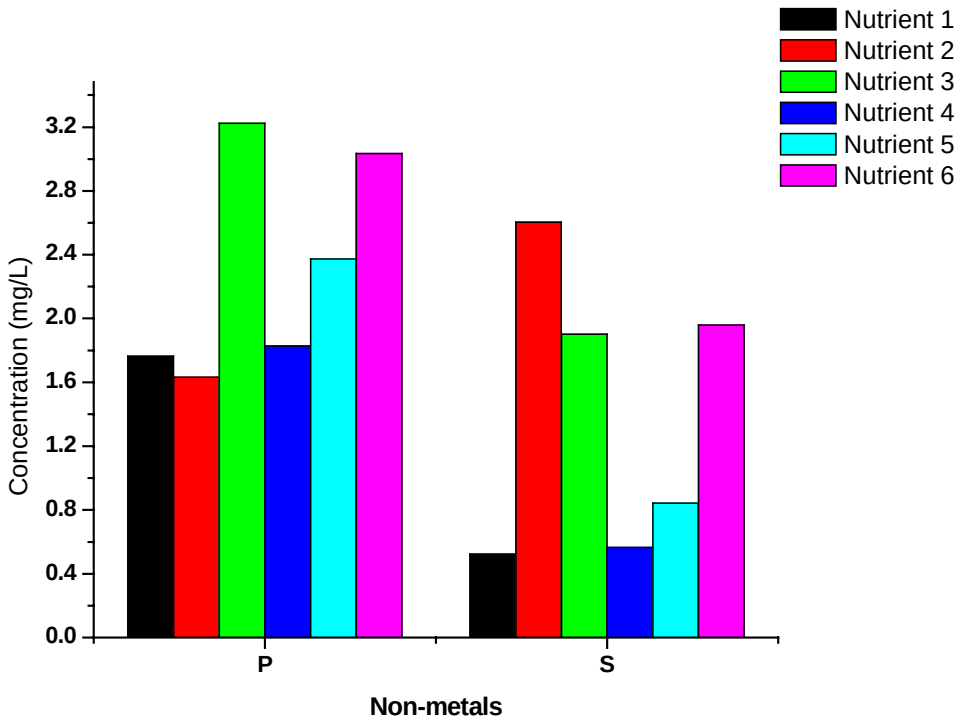

Figure 2.2: Elemental profile analysis of non-metals phosphorous and sulfur in commercial enological and malolactic nutrients. Mean concentrations are displayed for the recommended nutrient addition, which is 200 mg/L.

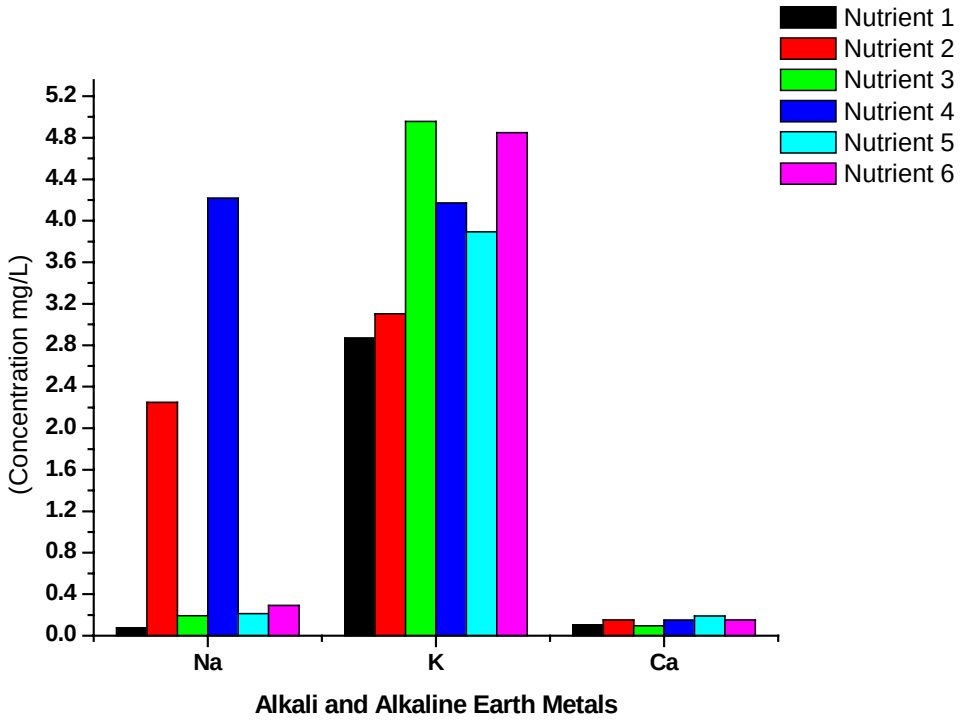

Figure 2.3: Results of elemental profile analysis for alkali and alkaline earth metals in commercial enological and malolactic nutrients. Mean concentrations are displayed for the recommended nutrient addition, which is 200 mg/L.

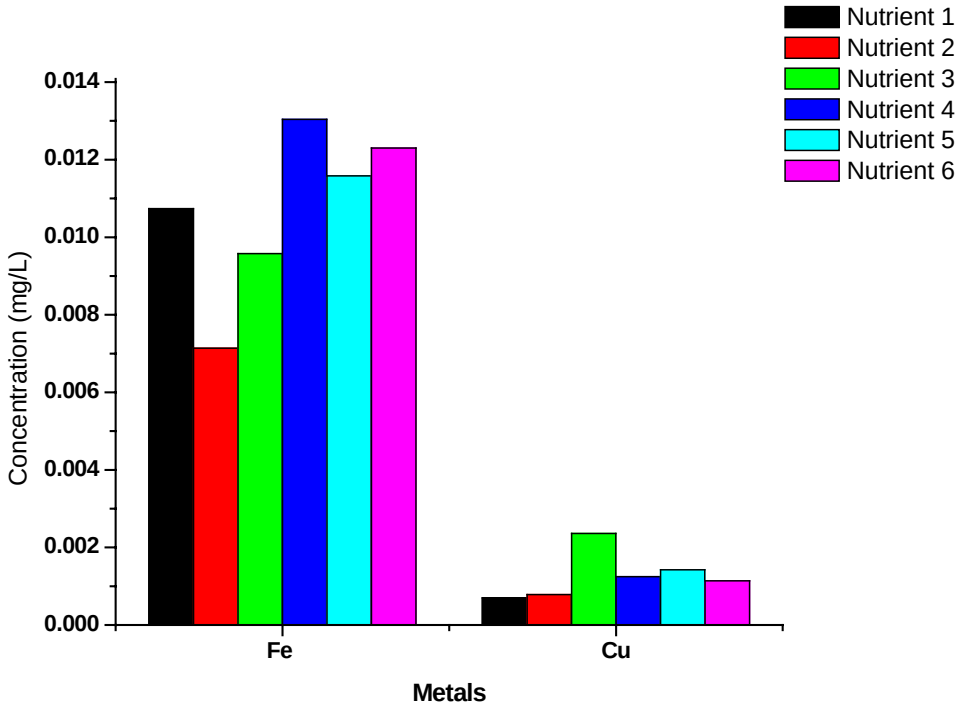

Figure 2.4: Results of elemental profile analysis for iron and copper in commercial enological and malolactic nutrients. Mean concentrations are displayed for the recommended dosage of nutrient, which is 200 mg/L.

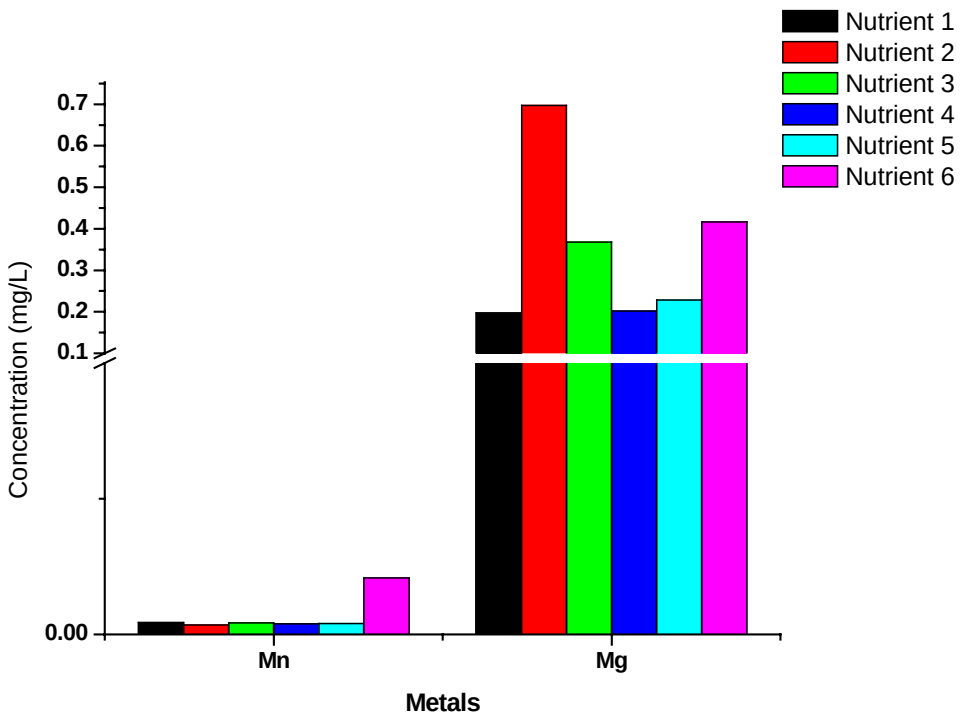

Figure 2.5: Results of elemental profile analysis for essential elements manganese and magnesium in commercial enological and malolactic nutrients. Mean concentrations are displayed for the recommended dosage of nutrient, which is 200 mg/L.

The nutrients contained trace quantities of lithium, beryllium, barium, and strontium (data not shown). Nutrients 5 and 6 had low levels of calcium, but high levels of potassium (Figure 2.3). All of the nutrients contained iron, copper, manganese, and magnesium, but in low quantities (Figures 2.4-2.5). Levels of arsenic were highest among the four toxic elements displayed (Figure 2.1), and was found in all nutrients, except for Nutrient 4. Nutrient 3 contained the highest levels of cobalt, lead, and arsenic, but did not contain any nickel. All of the nutrients contained high levels of phosphorous and sulfur (Figure 2.2). Nutrients 5 and 6 had low levels of calcium, but high levels of potassium (Figure 2.3).

Glutathione Content

The results from the first glutathione analysis (Analysis 1), show relatively low concentrations of reduced glutathione (GSH), with the exception of Nutrient 6 which exhibited the highest

concentration of GSH (Table 2.5). The second analysis (Analysis 2) shows a decrease in the concentration of GSH in Nutrient 6, reflecting a modifiation in manufacturer formula after initial anlayses showed a that GSH concentration exceeded permitted levels.

Nutrient	GSH (mg/L) Analysis 1	+/- Standard Error	GSH (mg/L) Analysis 2	+/- Standard Error
1	Nd	-	0.13	0
2	0.07	0.02	0.14	0.01
3	0.57	0.01	0.94	0.01
4	Nd	-	nd	-
5	Nd	-	0.23	0
6	55.35	0.72	0.36	0.01

Table 2.5: Results of glutathione content analysis before and after the adjustment of Nutrient 6 formula for glutathione. Mean concentrations are displayed for the recommended dosage of nutrient, which is 200 mg/L.

Free Amino Nitrogen (FAN) and Ammonium Content

Nutrient 6 exhibited the highest concentration of free amino nitrogen, having almost ten times the amount of nitrogen as Nutrient 1. Nutrient 5 exhibited the highest concentration of ammonium (Table 2.6).

Nutrient	Free Amino Nitrogen (FAN) (mg/L)	Ammonium (mg/L)
1	0.283	0.073
2	0.708	0.081
3	0.632	0.036
4	0.980	0.048
5	0.480	0.399
6	2.495	0.174

Table 2.6: Free amino nitrogen (FAN) and ammonium for six enological and malolactic nutrients. Values shown represent concentrations in the recommended dosage of nutrient, which is 200 mg/L.

Free Amino Acids

During the UHPLC analyses, amino acids such as serine, threonine, glycine, were only quanitifed for some of the nutrients, as dictated by peak shape and resolution. Cysteine and ornithine could not be quantified because of poor peak resolution. All nutrients exhibited very

high levels of glutamic acid, alanine, and proline. However, proline was not detected in Nutrient 3, and citrulline was not detected in Nutrient 4. Nutrient 6 had the highest concentration of all amino acids (Table 2.7). During the ion-exchange analysis, cysteine was not detected in any of the nutrients, asparagine was only detected in Nutrient 6, and tryptophan and cysteine were not quantified because of method limitations (Table 2.8). The ion-exchange method results also displayed high concentrations for glutamic acid and alanine. In general, Glutamic acid was the amino acid which exhibited the highest concentration in the recommended dosage of nutrient for both types of analyses. Concentrations of all amino acids varied by analysis; UHPLC results ranged from 0.012 mg/L to 3.325 mg/L, while ion exchange analysis results ranged from 0.022 mg/L to 3.584 mg/L. The ion-echange method results for the total amino acid analysis also displayed high concentrations for glutamic and aspartic acid equivalents and alanine (Table 2.9).

Amino Acid (mg/L)	Nutrient 1	Nutrient 2	Nutrient 3	Nutrient 4	Nutrient 5	Nutrient 6
Aspartic Acid	0.573	0.371	0.061	0.437	0.130	1.111
Glutamic Acid	3.141	2.126	2.276	2.325	1.445	3.325
Serine		0.272		0.115		1.579
Histidine	0.284	0.204	0.315	0.229	0.284	0.575
Glycine	0.544		0.012	0.137	0.128	1.106
Threonine		0.172	0.221	0.267	0.209	0.710
Arginine	0.488	0.448	0.418	0.199	0.242	1.032
Alanine	1.198	1.339	1.665	1.055	0.972	2.469
Tyrosine	0.226	0.293	0.127	0.261	0.160	0.669
Valine	0.407	0.622	0.363	0.469	0.281	1.262
Methionine	0.107	0.157	0.117	0.133	0.129	0.358
Phenylalanine	0.347	0.294	0.309	0.478		1.163
Isoleucine	0.243	0.382	0.152	0.355	0.176	0.930
Leucine	0.233	0.515	0.148	0.515	0.169	1.361
Lysine	0.698	1.170	0.437	0.560	0.397	1.369
Proline	0.867	1.162	Nd	0.866	1.238	1.445
Tryptophan	0.146	0.183	0.171	0.174	0.232	0.396
Citrulline	0.077	0.104	0.126	nd	0.181	0.142
Glutamine	0.422	0.243	0.777	0.098	0.394	0.786
Asparagine	0.137	0.228	0.248	0.099		0.927

Table 2.7: Results of free amino acid analysis by UHPLC for the six enological and malolactic nutrients (nd = not detected, blank space = unable to quantify based on peak shape). Mean concentrations are displayed for the recommended nutrient addition, which is 200 mg/L.

Amino Acid (mg/L)	Nutrient 1	Nutrient 2	Nutrient 3	Nutrient 4	Nutrient 5	Nutrient 6
Aspartic Acid	0.363	0.330	0.296	0.388	0.167	1.092
Glutamic Acid	2.953	1.789	2.764	1.883	2.226	3.584
Serine	0.098	0.226	0.184	0.226	0.177	0.801
Histidine	0.037	0.022	0.048	nd	0.034	0.192
Glycine	0.140	0.196	0.075	0.208	0.102	0.463
Threonine	0.077	0.251	0.104	0.235	0.148	0.656
Arginine	0.352	0.269	0.339	0.051	0.362	0.833
Alanine	1.097	1.148	1.984	1.021	1.392	2.337
Tyrosine	0.122	0.217	Nd	0.208	2.831	0.648
Valine	0.251	0.499	0.383	0.385	0.242	1.104
Methionine	nd	0.107	Nd	0.089	nd	0.365
Phenylalanine	nd	0.321	0.127	0.249	0.104	0.813
Isoleucine	0.150	0.344	0.119	0.287	0.144	0.826
Leucine	0.162	0.498	0.118	0.384	0.165	1.334
Lysine	0.202	0.318	0.136	0.249	0.136	0.708
Proline	0.268	0.374	0.154	0.302	0.543	0.617
Ornithine	0.396	0.282	0.828	0.181	0.223	0.531
Citrulline	nd	0.115	0.231	nd	nd	0.106
Glutamine	0.356	0.145	0.773	nd	0.521	0.737
Asparagine	nd	nd	Nd	nd	nd	0.506

Table 2.8: Results of free amino acid analysis by ion exchange chromotography for the six enological and malolactic nutrients. Values shown represent concentrations in the recommended nutrient addition, which is 200 mg/L. (nd = not detected)

Total Amino Acids

Amino Acid (mg/L)	Nutrient 1	Nutrient 2	Nutrient 3	Nutrient 4	Nutrient 5	Nutrient 6
Aspartic Acid	0.727	1.850	0.735	2.713	0.812	3.104
Glutamic Acid	3.466	4.054	6.141	6.058	3.876	7.117
Serine	0.177	0.605	0.347	0.845	0.317	1.188
Histidine	0.0846	0.280	0.111	0.374	0.118	0.566
Glycine	0.437	1.042	0.771	1.538	0.511	1.462
Threonine	0.173	0.694	0.300	0.910	0.320	1.267
Arginine	0.433	0.697	0.547	0.322	0.572	1.499
Alanine	1.048	1.670	2.099	2.167	1.598	2.890
Tyrosine	0.148	0.513	0.175	0.710	0.200	0.959
Valine	0.303	1.003	0.459	1.259	0.383	1.650
Methionine	0.022	0.084	nd	0.133	nd	0.134
Phenylalanine	0.140	0.631	0.163	0.780	0.187	1.112
Isoleucine	0.171	0.739	0.206	0.920	0.232	1.330
Leucine	0.220	1.059	0.268	1.334	0.311	1.895
Lysine	0.385	1.285	0.455	1.545	0.468	2.202
Proline	0.338	0.852	0.315	1.071	0.676	1.214

Table 2.9: Results of total amino acid analysis by ion exchange chromotography for the six enological and malolactic nutrients. Values shown represent concentrations in the recommended nutrient addition, which is 200 mg/L. (nd = not detected) . (Aspartic and glutamic acid values represent aspartic and glutamic acid equivalents.)

Vitamins and Fatty Acids

Of 25 compounds (11 vitamins and 14 fatty acids), only five vitamins and five fatty acids were recovered during the analysis. Gamma-Linoleic acid and alpha-Linoleic acid had the same retention time and mass and hence could not be separated. Overall, quantities of both vitamins and fatty acids were very low among the 6 commerical nutrients analyzed. Riboflavin was the vitamin exhibiting the highest concentration among the nutrients. All nutrients were also high in palmitoleic and oleic acids (Table 2.10).

	Nutrient 1	Nutrient 2	Nutrient 3	Nutrient 4	Nutrient 5	Nutrient 6
Vitamins and Derivatives of Vitamins (mg/L)						
Riboflavin	0.044	0.033	0.050	0.017	0.049	0.115
Niacin	0.005	0.010	0.0001	0.006	0.003	0.002
Nictinamide (Derivative of Niacin)	0.004	0.002	0.004	0	0.004	0.001
Pantothenic Acid	0.007	0.004	0.006	0.0002	0.003	0.004
Pyridoxine	0.0003	0.0003	0.0005	0.0003	0.0007	0.0005
Fatty Acids (mg/L)						
Palmitic Acid	0	0.733	0	0.244	0	0
Palmitoleic Acid	0.417	1.65	0.487	1.33	0.472	0.299
Stearic Acid	0	0.454	0	0.181	0	0
Oleic Acid	0.412	1.87	0.415	1.75	0.446	0.429
Linolic Acid	0	0.104	0.082	0	0	0

Table 2.10: Results of the vitamin and fatty acid analyses for six commercial enological and malolactic nutrients at the recommended nutrient addition, which is 200 mg/L.

Growth Studies

Preliminary Work

Results displayed in Figure 6 suggest that Nutrient 5 produced the highest level of growth for all strains of LAB, except for the lower inoculum level for *L. plantarum* 1122, which exhibited . slightly growth rates and levels with Nutrient 3. According to the Ymax values of each growth curve, five of the six inoculations exhibited the highest Ymax value when exposed to Nutrient 5.

The "scaled up" version of the preliminary growth study was not successful. Over a period exceeding 2 months no growth was detected, even after re-inoculation.

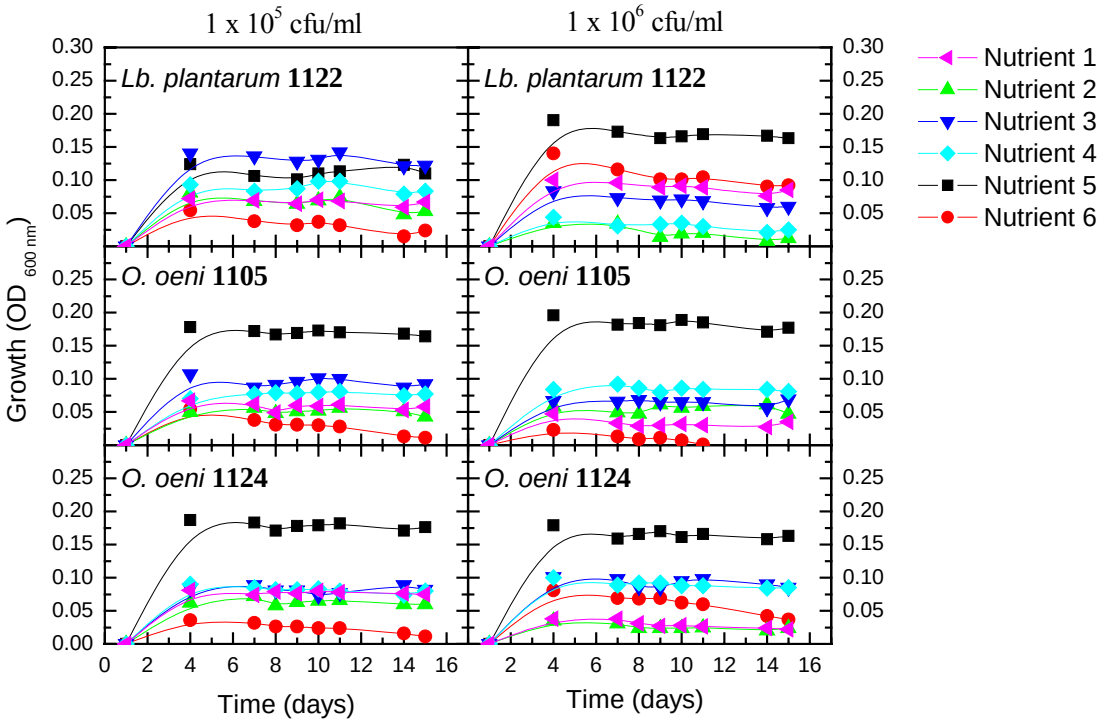

Figure 2.6: Growth curves from the preliminary growth study with three strains of wine lactic bacteria in combination with the six enological and malolactic nutrients.

Growth Study with Modified Model Wine

For the fermenations which were inoculated with *O. oeni* 1124 (Figure 2.9) and *Lb. plantarum* 1122 (Figure 2.7), the growth of those with the additon of Nutrients 1, 3, and 6 was metabolically maintained throughout the course of fermentation, while the cell counts for the rest of the bacteria decreased. For those inoculated with *O. oeni* 1105, the cell counts of all cultivations decreased thoughout the course of fermentation. Those with the addition of Nutrient 4 and the control, which had no nutrient addition, displayed the least amount of growth and cell counts decreased the most dramatically for all strains (Figures 2.7-2.9).

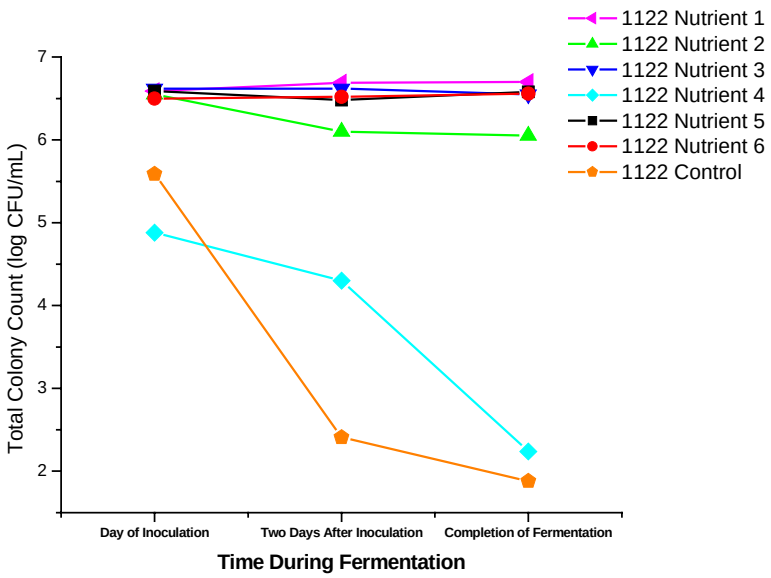

Figure 2.7: Mean colony count results of fermentation with the modified model wine for *Lb. plantarum* 1122 in combination with the six enological and malolactic nutrients. Cultivations with Nutrient 4 and the control were plated even though malolactic fermentation was not complete.

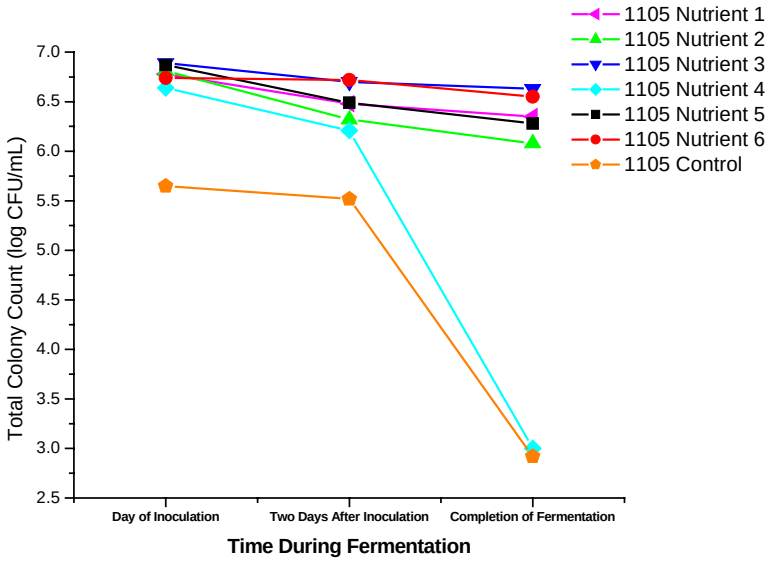

Figure 2.8: Mean colony count results of fermentation with the modified model wine for *O.oeni* 1105 in combination with the six enological and malolactic nutrients. Cultivations with Nutrient 4 and the control were plated even though malolactic fermentation was not complete.

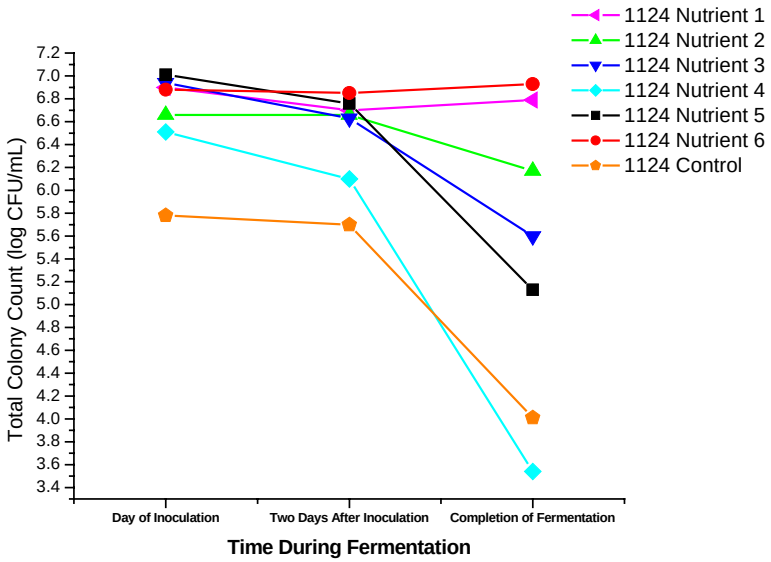

Figure 2.9: Mean colony count results of fermentation with the modified model wine for *O.oeni* 1124 in combination with the six enological and malolactic nutrients. Cultivations with Nutrient 4 and the control were plated even though malolactic fermentation was not complete.

L-Malic Acid Assay

The concentration of L-Malic acid in all cultivations at the start of each fermentation with modified model wine was 3.5 g/L. On the third day of the fermentation, cultivations in which Nutrient 3 and Nutrient 6 were added for *O.oeni* 1124, *O. oeni* 1105, and *Lb. plantarum* 1122, exhibited a complete metabolism of L-malic acid. By the sixth day of the fermentation, all cultivations except the control and those in which Nutrient 4 was added were complete. The control and cultivations in which Nutrient 4 was added, did not completely metabolize L-malic acid during the test period, but were plated for viable cell counts after 3 weeks to monitor growth.

Free Amino Acids in Post-Fermentation Supernatant Samples

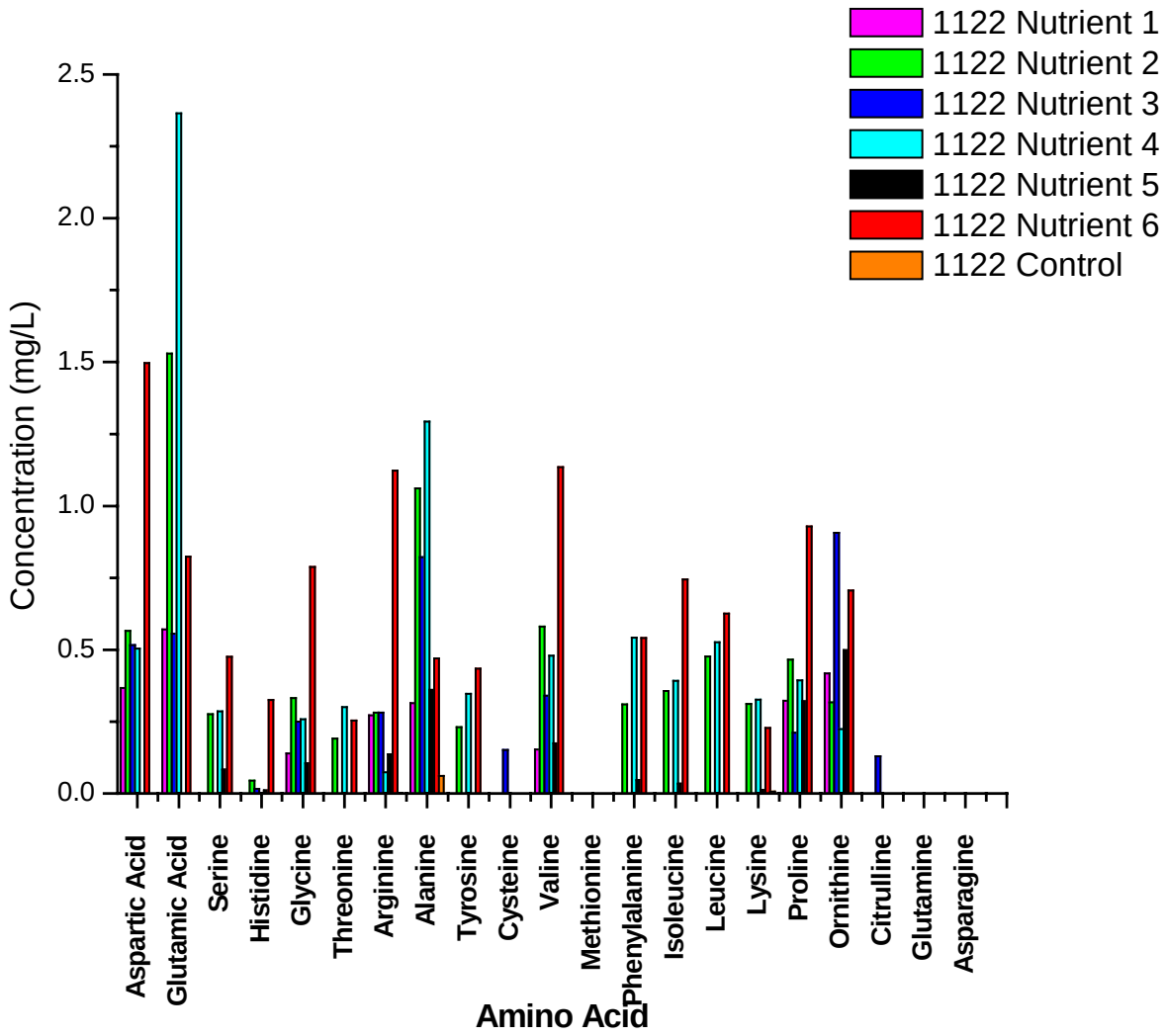

Figure 2.10: Mean free amino acid concentrations in supernatant samples following fermentation in modified model wine by *Lb. plantarum* 1122 in combination with the six enological and malolactic nutrients.

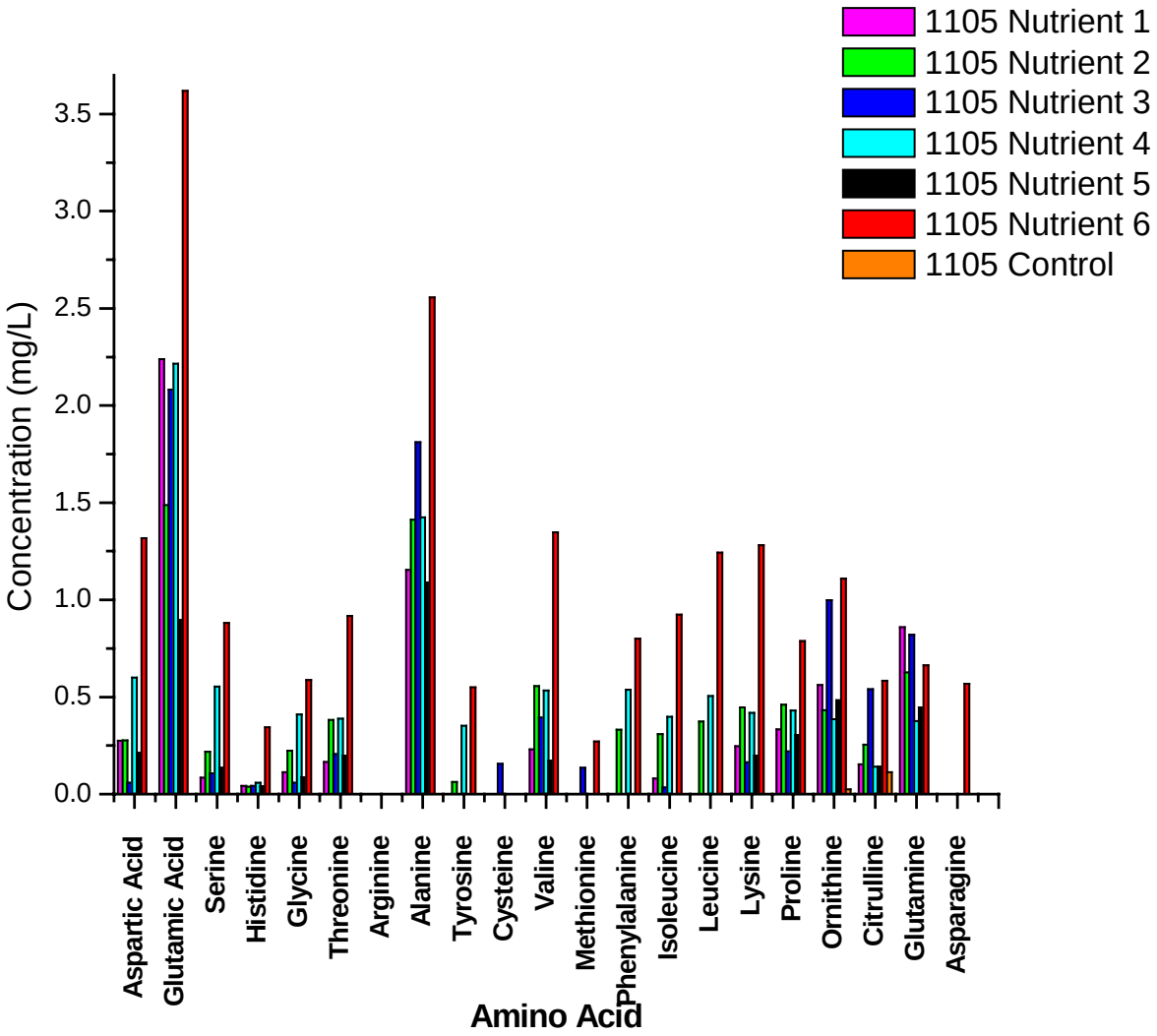

Figure 2.11: Mean free amino acid concentrations in supernatant samples following fermentation in modified model wine by *O.oeni* 1105 in combination with the six enological of malolactic nutrients.

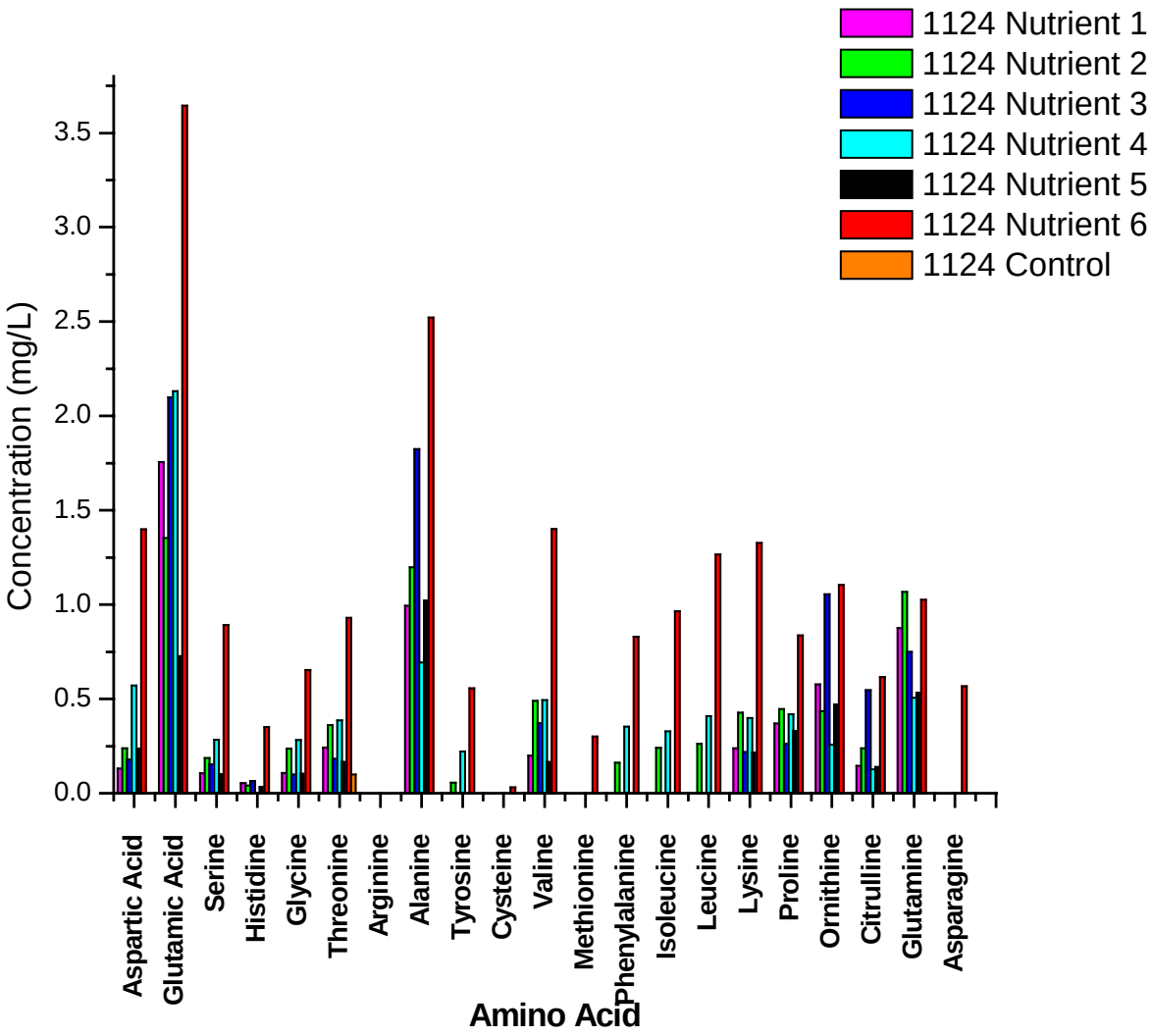

Figure 2.12: Mean free amino acid concentrations in supernatant samples following fermentation in modified model wine by *O.oeni* 1124 in combination with the six enological or malolactic nutrients.

Supernatant samples for the control fermentations of the three LAB strains displayed little to no amino acids. It is interesting to note that some amino acid concentrations in the nutrients themselves (Table 2.8), were lower than concentrations found in post-fermentation supernatant samples (Figures 2.10-2.12). In addition, most supernatant samples were still high in glutamic acid and alanine at the completion of the fermentation. Fermentations inoculated with *O.oeni* 1124 in combination with Nutrients 1-5 exhibited the lowest concentrations of amino acids in general.

Total Amino Acids in Post-Fermentation Supernatant Samples

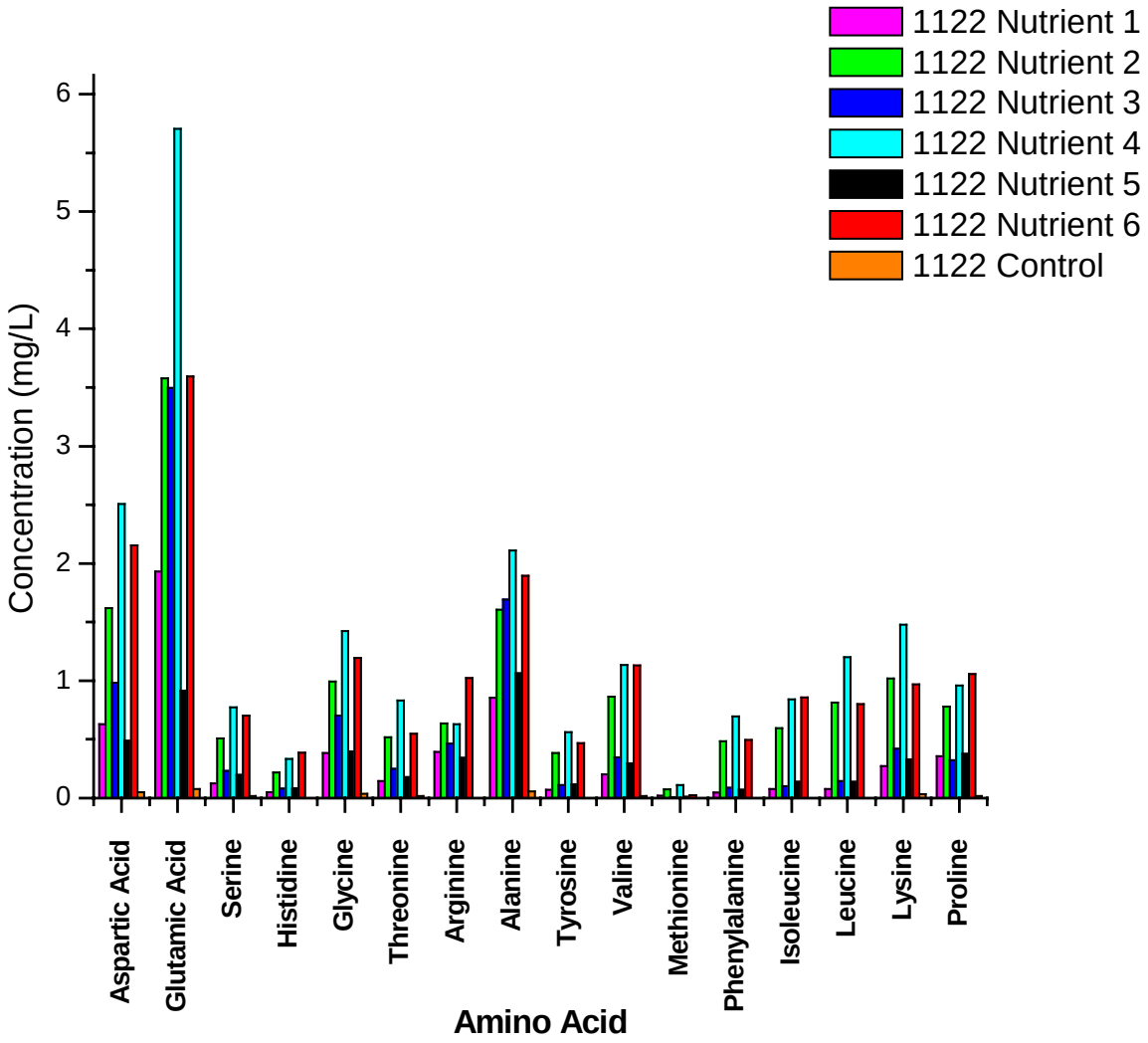

Figure 2.13: Mean total amino acid concentrations in supernatant samples following fermentation in modified model wine by *Lb. plantarum* 1122 in combination with the six enological and malolactic nutrients. (Aspartic and glutamic acid values represent aspartic and glutamic acid equivalents.)

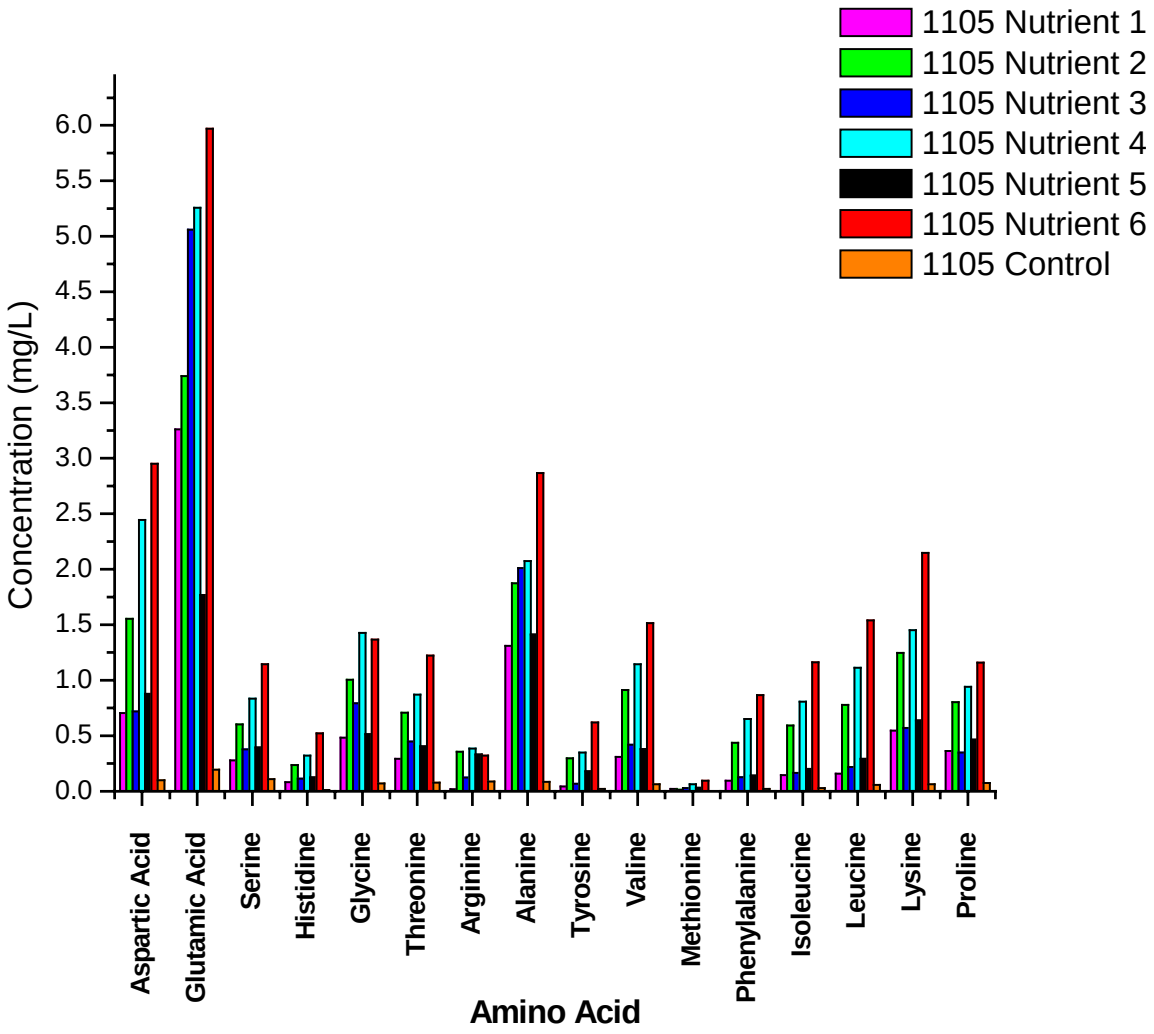

Figure 2.14: Mean total amino acid concentrations in supernatant samples following fermentation in modified model wine by *O.oeni* 1105 in combination with the six enological and malolactic nutrients. (Aspartic and glutamic acid values represent aspartic and glutamic acid equivalents.)

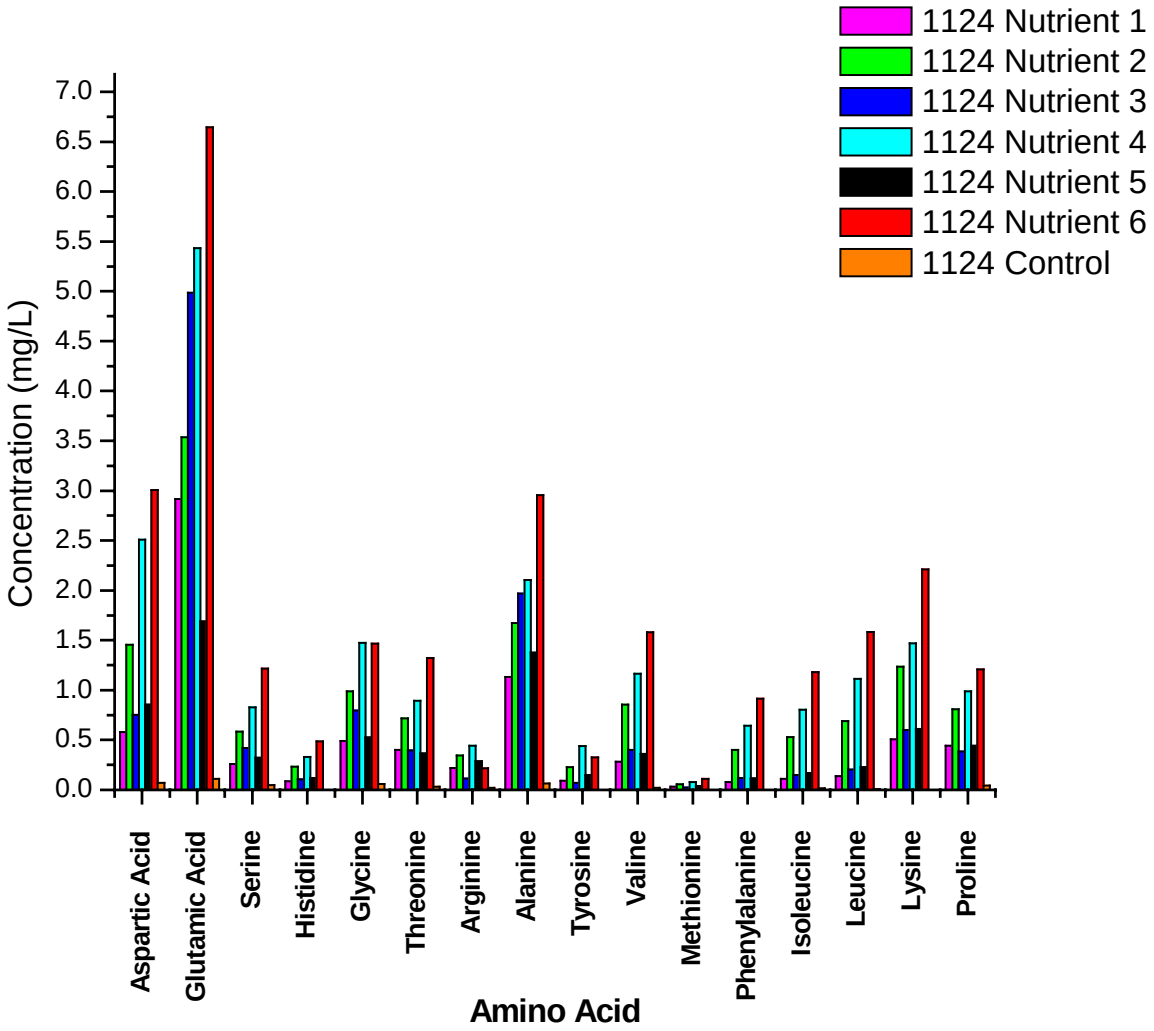

Figure 2.15: Mean total amino acid concentrations in supernatant samples following fermentation in modified model wine by *O.oeni* 1124 in combination with the six enological and malolactic nutrients. (Aspartic and glutamic acid values represent aspartic and glutamic acid equivalents.)

Nutrients 4 and 6 had the highest concentrations of total amino acids in post-fermentation supernatant samples across bacterial strains. Nutrient 4 was not degraded at all, likely because of bacterial die-off for all strains. Nutrient 6 had an excess of both free and total amino acids present, which was shown to be beneficial to all strains.

Statistics

Results from the ANOVA statistical tests with a p-value less than 0.05 were considered significant. Among the concentrations of elements, glutathione, and amino acids (results from

the UHPLC method) in the nutrients, only concentrations of phosphorous, potassium, and leucine showed a difference with p-values of 0.0262, 0.0166, and 0.0001 respectively . The ANOVA test performed for the growth study using the the modified model wine suggested that bacterial strain, nutrient, stage of fermentation, and combination of bacteria/nutrient/stage resulted in different growth levels (Table 2.11).

Source	Number of Parameters	Prob>F (p-value)
Bacteria	2	<0.0001
Nutrient	6	<0.0001
Stage in Fermentation	2	<0.0001
Bacteria/Nutrient	12	<0.0001
Bacteria/Stage	4	0.0108
Nutrient/Stage	12	<0.0001
Bacteria/Nutrient/Stage	24	0.0010

Table 2.11: Results of ANOVA Fixed Effects Test for differences in growth level among variables and combination of variables.

DISCUSSION

As expected, the six enological and malolactic nutrients tested in this study differed in composition. Although the moisture content was not high in any of the nutrients, Nutrient 6 had the highest percentage of moisture, likely due to hygroscopy or the strain of yeast used for production of this particular nutrient. Nutrient 5 had a higher turbidity and more non-solubles, which may limit its efficacy. A higher turbidity could suggest that this product cannot dissolve completely in wine, making it possible that wine lactic acid bacteria are not properly absorbing the nutritional compounds present.

Concentrations of GSH were very low in all of the enological nutrients, and may not contribute to nutritional requirements or the oxidative protection of wines. Analysis 1 (Table 2.5) displayed a very high level (55.35 mg/L) of GSH in Nutrient 6, but this level was later reduced in the reformulated product due to the legality of glutathione used in winemaking. Levels of

ammonium were also low overall. Lactic acid bacteria do not utilize ammonium, but it can still be an important factor to consider for enological nutrient quality, as it is metabolized by yeast. Higher levels of ammonium in nutrients could be indicative of high additions of diammonium phosphate during the production of the enological nutrient.

Low free amino nitrogen levels coincide with low free amino acids levels displayed in the results of these analyses. According to the UHPLC free amino acid analysis, all nutrients displayed high concentrations of glutamic acid, alanine, and proline, except for Nutrient 3, in which proline was not detected for (Table 2.7). Of these three amino acids, proline has been shown by some to be an essential nutritional requirement for wine lactic acid bacteria (Terrade and Mira de Orduña, 2009). The nutrients did not contain high levels of arginine, or any of the branched amino acids such as isoleucine, leucine and valine, which are known to be essential. Nutrient 6 had the highest concentrations of most amino acids, which could give this nutrient an advantage with regards to stimulating growth. High levels of amino acids, especially tyrosine and lysine (Marco, *et al.,* 2006) may also be of concern, however, as they can be precursors to biogenic amines. In general, differences in amino acid content may be dictated by the yeast strains used for production of these nutrients.

Analysis by ion-exchange chromotography for free amino acids (Table 2.8) returned overall lower concentrations of amino acids in all samples when compared to UHPLC free amino acid analysis. It would be expected, however, that the concentations found in the nutrients themselves would be greater than the concentrations found in the supernatant samples due to degradation of amino acids by the wine lactic acid bacteria during fermentation, but this was not true in all cases. Also, while it would be expected that levels of total amino acids determined by ion-exchange chromotography would be higher than the levels of free amino acids in both the

nutrients themselves (Table 2.9) and in the supernatant samples (Figures 2.13-2.15), these levels were not much higher. This similiarity could indicate the destruction of bound amino acids during the production of the yeast extracts used for nutrient production.

Overall, quantities of both vitamins and fatty acids were very low among the nutrients analyzed. Riboflavin, which exhibited the highest concentration among the supplemented nutrients, is considered to be an essential vitamin for LAB (König and Berkelmann-Löhnertz, 2009). All nutrients were also high in palmitoleic ($C_{16:1}$) and oleic ($C_{18:1}$) acids (Table 2.10). High levels of fatty acids such as C_{18}, $C_{18:1}$, and $C_{18:2}$ have been shown to increase cell biomass during lactic acid bacteria fermentation (Guilloux-Benatier *et al.*,1998).

The growth study using modified model wine exhibited clear differences in growth based on the nutrient added. For all three bacterial strains, the control fermentations and those performed with Nutrient 4 showed the lowest bacterial growth. As analyses showed that the modified wine lacked any nitrogen source, it is not surprising that the control fermentations did not support growth. Nutrient 4, an enological nutrient intended to support yeast growth during alcoholic fermentation, may lack essential compounds needed to stimulate LAB for efficient MLF. Specifically, Nutrient 4 was found to contain low levels of glutathione and also of ribolflavin. Glutathione is important for bacterial stress protection while riboflavin is an essential nutritent for wine lactic acid bactera (Terrade and Mira de Orduña, 2009). The high levels of all amino acids remaining in the post-fermentation supernatant further suggest that Nutrient 4 was not efficiently utilized for bacterial growth.

In contrast, the growth studies suggested that bacteria were most metabolically active in the presence of Nutrients 3 and 6 (Figures 2.7-2.9). Like Nutrient 4, Nutrient 3 is an enological nutrient intended to support yeast during alcoholic fermentation, but study data suggest that it

could help achieve an efficient MLF if added late in alcholic fermentation, prior to LAB starter culture addition. The ability to support MLF, however, also suggests that this nutrient may faciliate unwanted MLF fermentation if present at the end of an alcoholic fermentation. Nutrient 6, which is intended for MLF, displayed high levels in all amino acids, even post-fermentation. This may be of concern if remaining nutrients are make the wine more hospitable to spoilage microorganisms.

In general, results of these compositional analyses present valuable information to those in the winemaking industry. Enological and malolactic nutrients are an investment for winemakers, and generally cost from $2.00 to $4.00 per 100 g, increasing wine price by $0.03 to $0.12 per bottle of production. However, there few sources of compositional data available to those who purchase enological nutrients, and it is uncertain what effects these enological nutrients have on malolactic fermentations in wines. Compositional analyses performed during production of enological nutrients would be an important step towards ensuring quality and allowing the development of standardized production practices. Further, providing compositional information to winemakers would allow better control over product choice, addition rates, and achievement of product goals.

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CHAPTER 3: RESEARCH CHALLENGES AND FUTURE WORK

RESEARCH CHALLENGES

ENOLOGICAL AND MALOLACTIC NUTRIENTS

The nutrient formulations themselves caused several problems for the analyses performed in these studies. Since the nutrients were heterogenous powders, some containing large particles, they dissolved poorly in solutions such as water or model wine. This left the model wine solutions very turbid, making it difficult to determine optimal density readings in the fermentations taking place in the model wine. Inconsistencies in compositional analyses data may also have been due to the insolubility of the nutrients.

COMPOSITIONAL ANALYSES

Since compositional analyses are not often performed on enological or malolactic nutrients, there was a lack of published data to compare the results generated from the 6 nutrient supplements. Although there is sometimes information on ingredients used to produce commercial nutrients on company websites, there is no specific compositional data, like there is for yeast extracts and other laboratory media components produced by microbiological media manufacturing companies. It is assumed that there are large batch-to-batch variations in the ingredients, such as yeast extracts, used to produce enological nutrients. This variation is likely responsible for inconsistencies in compositional analysis data (especially the UHPLC amino acid analysis). Sometimes, results of duplicate trials were not in the same range of concentration. Because of this, many analyses needed to be repeated.

Some of the compositional analyses were performed by outside certified laboratories because resources were not available for the analyses to be performed in house. These analyses

were sometimes costly, and results for fatty acid, vitamin, free amino acid, and total amino acid analyses displayed lower concentrations of analytes than expected.

For example, the UHPLC free amino acid results displayed much higher concentrations than the ion-exchange chromatography free amino acid results. This could be because of the variation in formulation of the nutrients themselves, or because of the different methods employed.

The UHPLC method for the analysis of free amino acids also displayed inconsistencies, even between analysis replicates, and the multiple derivatization reagents required for this method may have augmented this effect. Since all derivatization reagents were only stable for short periods of time, and there was no auto-sampler, all samples were derivatized by hand and separately, making it difficult to run many samples at one time.It is possible that the FMOC-Cl derivatization reagent, which was used for the separation of Proline, was being retained on the column, affecting peak shape dramatically and requiring frequent replacement of the guard cartridge. Further, in order to clearly distinguish the Proline peak, there was a need for a higher concentration of FMOC-Cl, exacerbating the column retention issue.

MICROBIOLOGY

The wine lactic acid bacteria (LAB) used for this research were easily stressed in the environment of the model wine solutions used. They were able to grow in an environment with an ethanol content of 8% (v/v) but not 12.5%(v/v), and a pH of 3.5, but not 3.2. The wine LAB were grown in AMRS broth and then transferred to model wine for the experiments. To avoid stressing the bacteria during transfer, the LAB could have been grown in numerous subcultures in increasing concentrations of ethanol and increasing acidity to allow for the cells to adapt to the harsh conditions.

Because the enological nutrients used for this research were composed mostly of yeast extract, the cultivations in model wine were easily contaminated by yeast, even in the presence of fungicide. Also, once contamination occurred, the yeast would compete with and inhibit the wine LAB.

FUTURE WORK

In order to get a better understanding of the composition of enological and malolactic nutrients used, it would be beneficial to perform all of the compositional analyses on more nutrients, especially on those intended for MLF. It would also be beneficial to repeat the same fermentations in actual wine rather than in model wine, to expose the wine LAB to a greater range of naturally occurring nutrients present after AF, in addition to the enological nutrients added to the fermentation. Further, it would also be interesting to investigate the performance of enological nutrients with different starter culture strains in a variety of wines, rather than a single model wine.

Perfecting an UHPLC method for amino acid analysis would be another future step for this project. A time-efficient method utilizing an auto-sampler to dervivatize the samples immediately before analysis would greatly improve this method, allowing for larger numbers of samples to be analyzed. If it were possible to rapidly analyze nutrients at a low cost, improving nutrient quality, standardizing quality control methods, and informing consumers about product content and use would be greatly simplified.